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# Chagasic antibodies induce cardiac COX-2/iNOS mRNA expression with $PGE_2$/NO production ☆

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**Abstract**

We demonstrate that serum IgG in chagasic patients interacting with the second extracellular loop of human cardiac $M_2$ muscarinic acetylcholine receptors ($M_2$ mAChR) trigger the production of $PGE_2$ and NO, that in turn induces COX-2/iNOS mRNA expression. An association between serum anti-$M_2$ peptide IgG, anti-cardiac membrane IgG and $PGE_2$ levels ($p<0.05$) in chagasic dysautonomic patients was observed. Thus, we establish that serum anti-mAChR autoantibodies and $PGE_2$ might be considered as early markers of Chagas' associated dysautonomia. Affinity purified anti-$M_2$ peptide IgG from chagasic sera, while stimulating myocardial $M_2$ mAChR, it exerts an increase on $PGE_2$ generation and NOS activity, as well as COX-2/iNOS isoforms mRNA expression. The expression of these genes is related with phosphoinositides (PIs), cGMP accumulation and PKC activity. Inhibition of these enzymes shows that chagasic autoantibodies up-regulation of COX-2/iNOS mRNA level is under the control of endogenous iNO/cGMP signaling system. These results provide a novel insight into the role that cholinoceptor antibodies play in the development of myocardial inflammation. To our knowledge, there has been no previous report showing that an antibody interacting with heart mAChR can act as expression inducer of proinflammatory mediators.


*Keywords:* Chagas' disease; Myocardial function; Autoantibodies; iNOS/COX-2 mRNA; Parasympathetic dysautonomia; $PGE_2$

## 1. Introduction

One of the theories proposed to explain chagasic myocarditis is that it is essentially an autoimmune disease triggered by the ability of the infectious agent (*Trypanosoma cruzi*) to trick the immune system into attacking itself, leading to heart damage [1–4]. Autoreactive lymphocytes and antibodies against sarcolemma epitopes have been described in chagasic sera [5–9]. During three decades, we have been studying the role of antibody and lymphocytes in the genesis of chagasic myocarditis. Our research has been based on the hypothesis that cell–cell and antibody–cell interactions, mediated through neurotransmitter receptors of myocardial fibers and immune competent cell, determine the genesis and evolution of myocarditis. It is our belief that anti-sarcolemmal antibodies and lymphocytes interacting with β-adrenergic and muscarinic cholinergic receptors (mAChR) trigger molecular alterations, inexorably leading to cardiac damage [7,10,11]. Moreover, the presence of serum

*Abbreviations:* AA, arachidonic acid; Anti-$M_2$ peptide IgG, IgG fraction subjected to affinity chromatography on the synthesized peptide; cGMP, cyclic guanosin mono phosphate; COX-1, cyclooxygenase 1; COX-2/iNOS mRNA, gene expression of COX-2/iNOS; COX-2, cyclooxygenase 2; ELISA, enzyme-linked immunosorbent assay; eNOS, endothelial nitric oxide synthase; iNOS, inducible nitric oxide synthase; $M_2$ mAChR, $M_2$ muscarinic acetylcholine receptors; nNOS, neuronal nitric oxide synthase; NO, nitric oxide; NOS, nitric oxide synthase; OD, optical density; $PGE_2$, prostaglandin $E_2$; PIs, phosphoinositides; PKC, protein kinase C; $PLA_2$, phospholipase $A_2$.

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antibodies against the second extracellular loop of human $M_2$ mAChR are associated with chagasic dysautonomic syndrome [8,12–14]. Fixation of chagasic autoantibodies to $M_2$ mAChR has important functional implication, since they limit not only the inotropic reserve of the myocardium, but also the effectiveness of the endogenous agonist [15]. These facts raise the question, how much neurotransmitter autoantibodies are implicated in the pathogenesis of chagasic myocarditis. In *T. cruzi* infected mice, the titer and frequency of serum antibodies against $M_2$ mAChR peptide were coincident with the increase of myocarditis index and with the severity of cardiac dysrhythmias. Moreover, infected mice treated *in vivo* with the $M_2$ mAChR peptide, reduced myocardial inflammation foci and improved cardiac dysfunction [15]. This has led to think that the damage and myocardial inflammation commonly seen in chronic Chagas' disease sufferers [1] might be a consequence of the production of proinflammatory mediators induced by antibody–mAChR interaction on cardiac sarcolemma.

Prostaglandins have been implicated in normal cellular processes as well as pathophysiological conditions, such as inflammation [16]. On the other hand, nitric oxide (NO) plays a key role in the pathophysiology of chronic inflammation and in the neurodegenerative process [17]. During inflammation, the generation of proinflammatory $PGE_2$ is nearly entirely driven by NO [17]. Endogenous NO has been found to switch on/off the COX pathway, and might induce expression of COXs [17]. In addition, NOS stimulation has been shown to be part of the signaling events involved in heart mAChR activation [18].

Taking these observations jointly, we focused our research on the possibility that, in addition to the direct alteration of cardiac contractility, the mAChR autoantibodies might contribute to the cardiac inflammatory reaction that characterizes chronic Chagas' disease [1]. We investigated whether the antibodies against mAChR can induce cholinergic NOS activation and $PGE_2$ generation, pointing to a novel insight into the mechanisms involved in the ability of anti-mAChR antibodies to act as an early inducer of myocardial COX-2 and iNOS gene expression. Moreover, this study provides evidences that anti-$M_2$ mAChR antibodies and $PGE_2$ in serum may serve as early markers of Chagas' associated dysautonomia among asymptomatic *T. cruzi* carriers.

## 2. Methods

### 2.1. Study population

We studied *T. cruzi* infected patients with positive serology residing in metropolitan Buenos Aires. These included asymptomatic subjects (with normal ECG, 24 h Holter record, ECHO and chest X-ray) and non-infected individuals (controls). The subjects were divided into three groups:

Group I: 65 asymptomatic patients with autonomic nervous system dysfunction, as shown by abnormal response to two or more of the following diagnostic tests: reduced diastolic blood pressure; poor rise in diastolic blood pressure in response to tilting test; less bradycardia during the straining phase of the Valsalva manoeuvre and less tachycardia during the releasing phase; hyporeactivity to the cough reflex test and hyporeactivity to the hyperventilation test. The criteria for diagnosis of dysautonomia were applied on the basis of previous reports [13].

Group II: 48 asymptomatic patients with normal cardiovascular response to autonomic nervous system tests.

Both groups (I and II) were not receiving any drug treatment. Serological studies for Chagas' disease (passive haemagglutination, enzyme-linked immunosorbent assay (ELISA) and immunofluorescence) were performed in all patients.

Group III: 70 healthy volunteers (control group) with negative serology and no evidence of cardiovascular disease, chronic systemic disease or acute viral or febrile disease. These subjects had normal ECGs and chest X-rays.

Table 1 shows the clinical characteristic and basic demographic between patient groups.

All the studies involving human subjects were conducted according to the Helsinki Declaration and informed consent was obtained from the subjects.

### 2.2. $M_2$ mAChR synthetic peptide

A 24-mer-peptide [V-R-T-V-E-D-G-E-C-Y-I-Q-F-F-S-N-A-A-V-T-F-G-T-A] corresponding to the sequence of the second extracellular loop of the human mAChR [residues 169–192] was synthesized as previously described [14].

### 2.3. Purification of the human IgG

The IgG fraction of human chagasic patients and normal individuals (control) was isolated by chromatography on DEAE-cellulose as previously described [14]. The degree of IgG purification was tested by SDS–PAGE (sodium dodecyl–sulphate polyacrylamide gel electrophoresis) and its concentration was determined by radial immunodiffusion assay.

Table 1
Characteristic of individuals and patients from different groups

| Characteristic of subjects and patients | Group I | Group II | Group III |
|---|---|---|---|
| Age (years) | 34±7.1 | 37±6.2 | 35±4.2 |
| Gender (% male) | 52 | 55 | 50 |
| EF (%) | 69±7.1 | 67±6.5 | 72±7.3 |
| NVPB (in 24 h) | 0–3 | 0–3 | 0–2 |
| Demographic Argentine North-West Area | 41 | 30 | 47 |
| Demographic Argentine North-East Area | 9 | 6 | 5 |
| Demographic Argentine Central Area | 15 | 12 | 18 |

Age and EF (ejection fraction) are expressed as mean±SEM. Number of ventricular premature beats (NVPB) is shown as the number in 24 h. Data of demographic argentine area are expressed as number of subjects in each group.

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### 2.4. Purification of antipeptide IgG by affinity chromatography

IgG fractions of 9 dysautonomic chagasic patients and 9 normal individuals were independently subjected to affinity chromatography on the synthesized peptide covalently linked to AffiGel 15 gel (Bio-Rat, Richmond, CA, USA) as previously described [19]. The IgG concentrations of both non-antipeptide antibodies (eluted from the column) and specific anti-muscarinic receptor peptide antibodies, were determined by radial immunodiffusion assay, and their immunological reactivity against the muscarinic receptor peptide was evaluated by enzyme immunoassay (ELISA). The concentration of the affinity purified anti-$M_2$ peptide IgG ($5\times10^{-7}$ M) that maximally increased optical density (OD, 2.9±0.3) values corresponds to $5\times10^{-6}$ M total IgG concentration (OD, 2.6±0.2). The non-anti-$M_1$ peptide IgG fraction eluted from the column showed OD values (0.27±0.06) similar to normal IgG (OD, 0.22±0.04). The normal IgG fraction purified by affinity column gave negative results (OD, 0.21±0.03).

### 2.5. Preparation of membranes

Male Wistar rats (3–4 months old) (obtained from the Pharmacology Unit, School of Dentistry, Buenos Aires University) were used throughout. The animals were cared for in accordance with the principles and guidelines of the National Institute of Health (NIH No. 8023, revised 4978). Atria was removed and placed on a glass plate containing a modified Krebs–Ringer bicarbonate (KRB) buffer, gassed with 5% $CO_2$ in oxygen pH 7.4; thus, fat, the large vessels, the connective tissue and blood were eliminated and membranes were prepared as previously described [15].

### 2.6. Enzyme-linked immunosorbent assay (ELISA)

Fifty microlitres (50 µl) of $M_2$ mAChR peptide solution (20 µg/ml) or cardiac membrane (3–5 mg/ml protein) in 0.1 M $Na_2CO_3$ buffer pH 9.6 was used to coat microtiter plates (Costar) at 4 °C overnight as previously described [19]. After blocking the wells, different dilutions of sera or purified IgG from chagasic patients and normal individuals were allowed to react with the antigens for 2 h at 37 °C. The wells were then thoroughly washed with Tween 20 in PBS. A 50 µl volume of goat anti-human IgG avidine–alkaline phosphatase 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 (OD) values were measured with an ELISA reader (Uniskan Laboratory System, Helsinky, Finland).

### 2.7. mRNA isolation and cDNA synthesis

Total RNA was extracted from rat atria by homogenization using guanidinium isothiocyanate method. 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 incubating rat atria in KRB gassed with 5% $CO_2$ in oxygen pH 7.4 at 37 °C for 60 min. In a selected tube, the reverse transcriptase was omitted.

Table 2
Oligonucleotides of primers for PCR

| Gene product | Sense | Antisense | Predicted size (bp) |
|---|---|---|---|
| iNOS | 5′ GAT CAA TAACCT GAA GCC CG 3′ | 5′ GCC CTT TTT TGC TCC ATA GG 3′ | 578 |
| nNOS | 5′ GCGGA GCAGA GCGGC CTTAT 3′ | 5′ TTTGGT GGGAG GACCG AGGG 3′ | 240 |
| eNOS | 5′ CCGCA CTTCT GTGCC TTTGC TC3′ | 5′GCTCG GGTGG ATTTGC TGCTCT 3′ | 360 |
| COX-1 | 5′ TAA GTA CCA GGT GCT GGA TGG 3′ | 5′ AGA TCG TCG AGA AGA GCA TCA 3′ | 160 |
| COX-2 | 5′ TCC AAT CGC TGT ACA AGC AG 3′ | 5′ TCC CCA AAG ATA GCA TCT GG 3′ | 242 |
| $G_3$PDH | 5′ ACCAC AGTCCA TGCCAT CAC 3′ | 5′ TCCAC CACCC TGTTG CTGTA 3′ | 452 |

Nitric oxide synthase (NOS) isoforms; glyceraldehydes-3-phosphate dehydrogenase ($G_3$PDH) and cyclooxygenase (COX) isoforms.

### 2.8. Quantitative PCR

Quantitation of iNOS and COX isoforms mRNA levels were performed by a method that involves simultaneous co-amplification of both the target cDNA and a reference template (MIMIC) with a single set of primers [20]. MIMIC for eNOS, nNOS, iNOS, COX-1, COX-2 and glyceraldehyde-3-phosphate dehydrogenase ($G_3$PDH) were constructed using a PCR MIMIC construction kit (Clontech Laboratories, Palo Alto, CA). The sequence of oligonucleotide primer pairs used for construction of MIMIC and amplification of eNOS, nNOS, iNOS and COX isoforms and $G_3$PDH mRNA 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 as previously described [20]. Levels of mRNA were calculated from the point of equal density of the sample and MIMIC PCR products [20]. COX and NOS mRNA levels were normalized with the levels of $G_3$PDH mRNA present in each sample, which served as control for variations in RNA purification and cDNA synthesis. The relative mRNA expression of COX and NOS in each group was compared with those from the respective normal group and reported as a percentage of normal.

### 2.9. $PGE_2$ and cyclic GMP assays

Rat atria (10 mg) were incubated for 60 min in 0.50 ml of KRB gassed with 5% $CO_2$ in oxygen at 37 °C. IgG was added 30 min before the end of incubation period and

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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). To determine cGMP production the cyclic GMP $^{125}$I-RIA KIT from Dupont New England Nuclear was employed. The $PGE_2$ results were expressed as picogram/milligram of tissue wet weight (pg/mg tissue wet wt) and cyclic (cGMP) in picomole/gram of tissue wet weight (pmol/g tissue wet wt).

### 2.10. Measurements of total labeled phosphoinositides (PIs)

Atria were incubated for 120 min at 37 °C in 0.5 ml of KRB gassed with 5% $CO_2$ in oxygen with 1 mCi *myo*-[$^3$H]-inositol ([$^3$H]MI) (specific activity 46.0 Ci/mmol) from Dupont/New England Nuclear, Boston, MA, USA. LiCl 10 mmol/l was added for inositol monophosphate accumulation, according to previously described [5]. Carbachol and IgGs were added 1 h before the end of incubation period and blockers 30 min before addition of reagents. Results corresponding to the second peak were expressed as the absolute values of the Simpson's equation.

### 2.11. Determination of nitric oxide synthase (NOS) activity

Nitric oxide synthase (NOS) activity was measured on rat atria by production of [U$^{-14}$C]-citrulline from [U$^{-14}$C] arginine as previously described [5], tissue were exposed to [U$^{-14}$C] arginine (0.5 μCi). Appropriate concentrations of enzymatic inhibitors were added and tissues were incubated for 20 min, and for an additional 1 h with carbachol or IgGs on carbogen at 37 °C. Tissues were then homogenized an Ultraturrax. After centrifugation at 2000 g for 10 min at 4 °C, supernatants were applied to 2 ml columns of Dowex AG-50 WX-8 (sodium form); [U$^{-14}$C]-citrulline was eluted with 3 ml of water and quantified by liquid scintillation counting. Measurement of basal NOS activity by the above-mentioned procedure was inhibited to a 95% by 0.5 mmol/l $N^G$-monomethyl-L-argininge (L-NMMA).

### 2.12. Protein kinase C (PKC) activity assay

Atria were incubated alone or in the presence of each concentrations of IgG, stimulant plus blockers or blockers alone for a total incubation time of 60 min in KRB solution at 30 °C and were frozen immediately in liquid nitrogen. PKC activity was purified from subcellular fractions as previously described [21]; and was assayed on both cytosolic and membrane preparations from atria. The PKC substrate peptide, MBP (4–14) from Life Technologies (Rochester, NY, USA) was used for measuring PKC activity purified from subcellular cardiac fractions, following the instructions of the PKC assay system of Life Technologies. PKC specificity was confirmed by means of the PKC pseudosubstrate inhibitor peptide PKC provided by Gibco (Calbad, CA, USA) [21]. The data were expressed in picomoles of phosphate incorporated into the substrate per minute and per milligram of protein (pmol/min/mg protein).

### 2.13. Contractile study

Animals were decapitated and atria were removed quickly and placed in a glass chamber containing KBB solution, pH 7.4, that was gassed with 5% $CO_2$ in oxygen at 30 °C. After a stabilization period of 30 min, spontaneous tension and frequency were recorded using a force transducer coupled to an ink-writing oscillograph, as previously described [18]. To obtain the maximum IgG effect, different concentrations of IgG were added to normal rat atria every 15 min.

### 2.14. Drugs

AF-DX 116, trifluoperazine (TFP), $N^G$-monomethyl-L-arginine (L-NMMA), aminoguanidine, calphostin C, were purchased from Sigma Chemical Company, Saint Louis, MO, USA; $N_5$-(1-iminoethyl)-L-ornithine dihydrochloride (L-NIO) and NZ (NZ) and 1*H*-[1,2,4]oxadiazolo[4,3-*a*] quinoxalin-1-one (ODQ), 4-(4-octadecylphenyl)-4-oxobutenoic acid (OBAA), 1-[4,5-bis(4-methoxyphenyl)-2thiazolyl] carbonyl-4-methylpiperazine hydrochloride (FR 122047) and 5-bromo-2-(4-flurophenyl)-3-[4-(methylsulfonyl)phenyl]-tiophene (DuP 697) were purchased from Tocris Cookson Inc.; Baldwin, MO, USA; 1–6–17β-3-methoxgestra-1,3,5 (10)-trien-17yl-aminohexyl-1-*H*-pirrole-2,5-dione (U-73122) from ICN Pharmaceuticals Inc., Aurora, OH, USA. Stock solutions were freshly prepared in the corresponding buffers. The drugs were diluted in the bath to achieve the final concentration stated in the text.

### 2.15. Statistical analysis

Analysis was performed using the computer program Graph Prism (Graph Pad, San Diego, CA, USA). Student's "*t*" test for unpaired values was used to determine the level of significance. The analysis of autoantibodies distribution was done using Fishers exact test. Pearson's analysis was applied to established correlation. Differences between means were considered significant if $p$ was equal to or less than 0.05.

## 3. Results

### 3.1. Detection of serum antibodies and $PGE_2$ levels

ELISA assays were performed to demonstrate if there is a correlation between serum IgG against $M_2$ mAChR synthetic peptide (A), serum IgG against cardiac membrane (B) as well as serum $PGE_2$ level (C) in dysautonomic chagasic patients. Fig. 1 shows the optical density (OD) values for each studied serum from seropositive patients with dysautonomia (group I) or without dysautonomia (group II) and normal subjects

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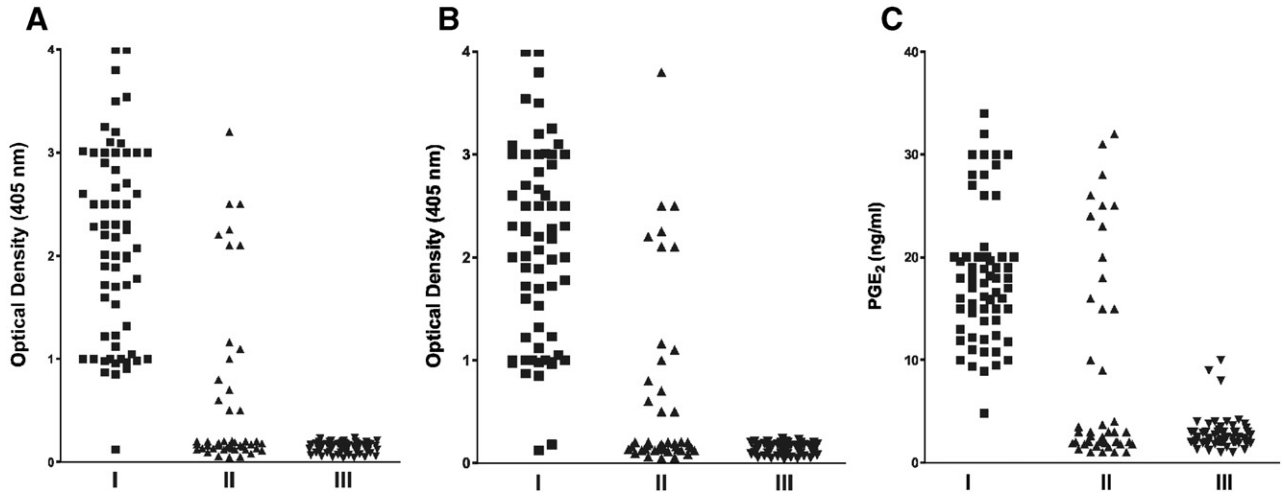


Fig. 1. Scatterogram showing immunoreactivity of circulating IgG antibodies against $M_2$ mAChR peptide (A) and cardiac membrane (B) and serum $PGE_2$ (C). The individual optical density values for each serum sample (1/30 dilution) from 65 chagasic asymptomatic patients with dysautonomia (group I) or 48 chagasic patients without dysautonomia (group II) and 70 normal individuals (group III). Cut off values of OD 0.240, 0.232 and 0.212 for anti-$M_2$ mAChR peptide and anti-cardiac membrane and serum $PGE_2$ respectively.

(group III). The OD values obtained with the group I sera were always >2 SD from those of normal individuals. It can be seen in Fig. 2 a positive correlation ($r=0.05$) between serum anti-$M_2$ peptide autoantibodies and anti-cell cardiac membrane IgG titers (A: Pearson $r=0.8252$, $p<0.0001$, $R^2$ 0.4875) of the individual sera from group I was observed. In Table 3, shows that the frequency of high serum $PGE_2$ levels, anti-cardiac membrane and anti-$M_2$ mAChR peptide autoantibodies was significantly higher in group I than in group II and group III ($p<0.0001$). Additionally, Table 3 shows the ability of the IgG from sera of the different groups to decrease contractility of isolated rat atria, showing an association between ELISA assays and the ability of the chagasic autoantibodies to trigger biological effect. Hence, the patients' sera, that reacted positively against cardiac $M_2$ mAChR showed high levels of $PGE_2$.

### 3.2. Stimulation of cardiac $PGE_2$ production and NOS activity by chagasic IgG

Knowing that the aminoacid sequence of rat and human $M_2$ synthetic peptide corresponding to the second extracellular loop of $M_2$ mAChR has a strong homology [15], we studied the mAChR-mediated effect of autoantibodies from chagasic patients on rat myocardium using the affinity purified anti-$M_2$ peptide antibodies (anti-$M_2$ peptide IgG)

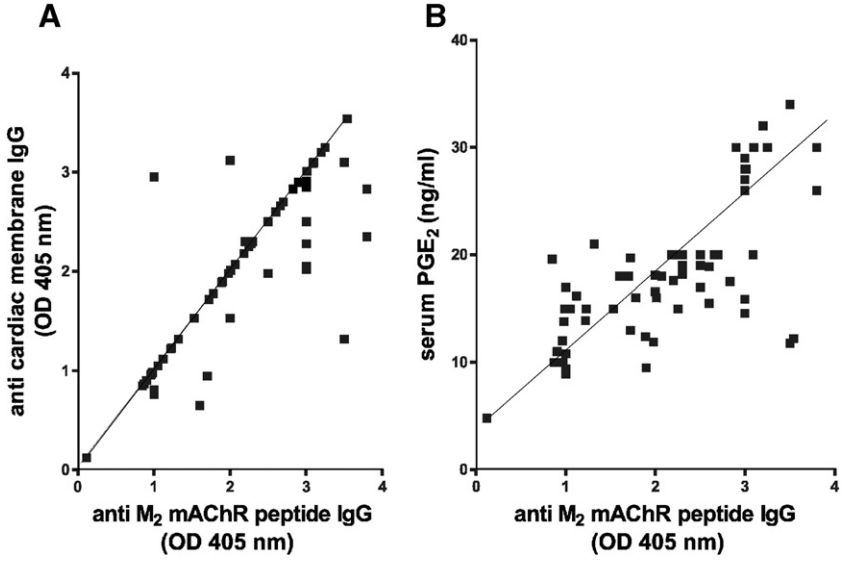


Fig. 2. Correlation between titers of serum anti-$M_2$ mAChR peptide IgG, anti-cardiac membrane IgG and serum $PGE_2$ levels in chagasic patients from group I. Anti-$M_2$ mAChR peptide IgG titers was plotted as a function of either anti-cardiac membrane IgG (A) or serum $PGE_2$ levels (B). The values correspond to the 64 chagasic dysautonomic patients.

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Table 3
Distribution of autoantibodies against cardiac membranes and $M_2$ mAChR peptide and serum $PGE_2$ tested by ELISA: association with biological data

| Groups | Number positive/total | | | |
|---|---|---|---|---|
| | Anti-$M_2$ peptide | Anti-cardiac membrane | Serum $PGE_2$ | Biological effect |
| I | 64/65 (98.46%)* | 64/65 (98.46%)* | 63/65 (97%)* | 63/65 (97%)* |
| II | 15/48 (31.50%) | 14/48 (29.1%) | 15/48 (31.5% ) | 13/48 (27%) |
| III | 0/70 (0%) | 1/70 (1.4%) | 3/70 (4.2%) | 3/70 (4.2%) |

Microtiter wells were coated with 50 μl of $M_2$ mAChR synthetic peptide (20 μg/ml) or cardiac membrane (3–5 mg/ml protein) and enzyme immunoassay (ELISA) was carried out in the presence of sera from group I (seropositive with dysautonomia), group II (seropositive without dysautonomia) and group III (normal subjects) as described in Methods. Optical density (OD) values more than 2 SD above the normal mean (0.150 ±0.11) were taken as positive. Cut off value of OD=0.240 and 0.232 for anti-$M_2$ peptide and anti-cardiac membrane respectively. $*p<0.0001$ versus group II and group III. Serum $PGE_2$ was measured by ELISA. Biological effect refers to decrease in contractility ($dF/dt$) of isolated atria exposed to $1\times10^{-7}$ M chagasic anti-$M_2$ mAChR IgG from different sera groups were used during 15 min as described in Methods.

from group I. Fig. 3 shows the ability of anti-$M_2$ peptide IgG to stimulate the production of $PGE_2$ (A) and NOS activity (B) in a concentration-dependent manner. The corresponding IgG fraction eluted from the column with $M_2$ peptide (non-peptide fraction) or the IgG from group III gave negative results. Fig. 3C demonstrates that under identical experimental conditions there is a significant correlation between anti-$M_2$ peptide stimulated production of $PGE_2$ and NOS activity (Pearson $r=0.9949$, $p<0.0004$, $R^2$ 0.9898, $n=5$). These results indicate that the activation of $M_2$ mAChR by chagasic IgG increase the production of $PGE_2$, may be as a result of stimulation of NOS activity.

### 3.3. NOS isoforms participation on chagasic IgG triggered $PGE_2$ production

To determine if endogenous NO participates in chagasic IgG-induced $PGE_2$ generation, isolated rat atria were incubated with particular NOS isoform inhibitors. As can be seen in Fig. 4A, the inhibition of all NOS isoenzymes with L-NMMA ($1\times10^{-5}$ M) decreased the stimulatory action of chagasic IgG on $PGE_2$ generation. Similarly, the specific inhibition of iNOS with aminoguanidine ($1\times10^{-5}$ M), or the nNOS inhibition with NZ ($1\times10^{-5}$ M) prevented generation of $PGE_2$ by the IgG. The inhibition of eNOS by L-NIO ($1\times10^{-5}$ M) had minor effect. Moreover, Fig. 4B shows that aminoguanidine ($1\times10^{-5}$ M), NZ ($1\times10^{-5}$ M) and L-NIO decreased chagasic IgG-stimulated NOS activity in different degrees. The iNOS inhibition was the most effective in both $PGE_2$ generation and NOS activity. As control, L-arginine ($5\times10^{-5}$ M) reversed the L-NMMA effect (data not shown). These results indicate that chagasic IgG–$M_2$ mAChR stimulation may trigger production of $PGE_2$ as a result of increase in NO with major participation of iNOS.

### 3.4. NOS enzymatic pathway participated in chagasic IgG-stimulated $PGE_2$ production

To investigate if endogenous NO signaling system participates in the increase of $PGE_2$ production by chagasic IgG, the effect of selective inhibitors of enzymatic pathways commonly associated with $M_2$ mAChR activation was studied. Fig. 5 shows that the inhibition of PLC by U-73122 ($5\times10^{-6}$ M), the PKC inhibition by calphostin C ($5\times10^{-9}$ M) and soluble guanylate cyclase inhibition by ODQ ($5\times10^{-6}$ M), attenuated the increase in $PGE_2$ production by chagasic antibody.

In order to assess the action of chagasic antibody on enzymatic pathways coupled to $M_2$ mAChR; PI and cGMP accumulation, as well as PKC and NOS activity were measured in atria exposed to anti-$M_2$ peptide IgG. As shown in Table 4, chagasic antibody increased PI and cGMP accumulation, as well as PKC translocation and NOS activity, while normal IgG had no effect. These effects resembled those of the authentic agonist carbachol. Furthermore the maximal increment induced by chagasic IgG was

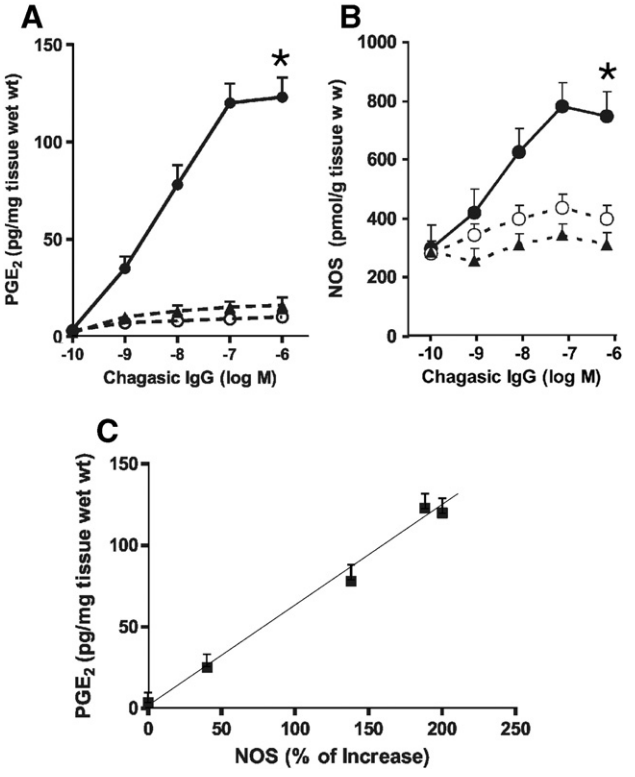


Fig. 3. Dose–response curve of anti-$M_2$ peptide chagasic IgG (●), normal IgG (○) and chagasic IgG eluted from $M_2$ peptide (non-peptide fraction) (▲) induced stimulation of $PGE_2$ accumulation (A) and NOS activity (B). Atria were incubated with each concentration of the reactive for 2 h and $PGE_2$ and NOS activity were assayed as described in Methods. Values are mean±SEM of nine chagasic IgG or nine normal IgG. Experiments were performed in duplicates. $*p<0.001$ versus normal IgG or non-peptide fraction of chagasic IgG. (C) Correlation in the stimulatory effect of anti-$M_2$ peptide chagasic IgG from $1\times10^{-9}$ to $1\times10^{-6}$ M on $PGE_2$ and NOS $PGE_2$ was plotted as a function of NOS.

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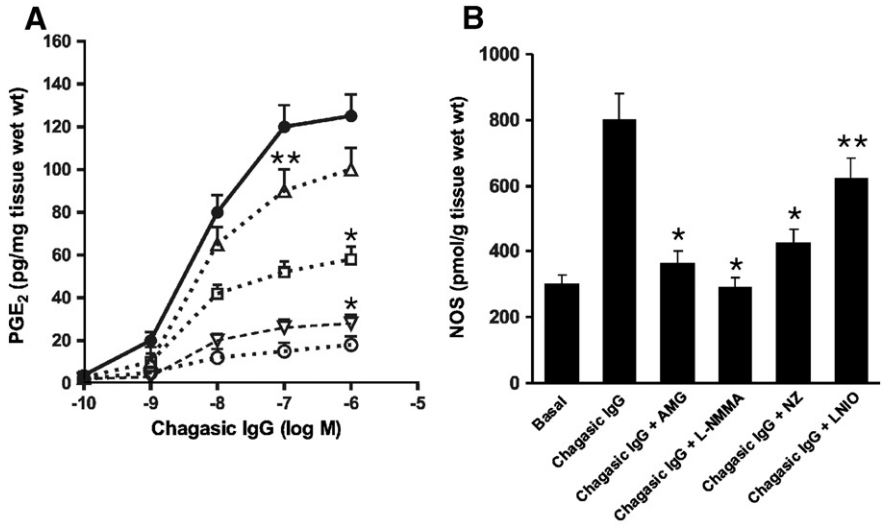


Fig. 4. (A) Concentration–response curve of anti-$M_2$ peptide chagasic IgG (●) induced $PGE_2$ accumulation effect in absence (●), or in presence of $1\times10^{-4}$ M L-NMMA (○), $1\times10^{-5}$ M aminoguanidine (▽), L-NIO, $1\times10^{-5}$ M (Δ), or $1\times10^{-5}$ M NZ (□). Values are mean±SEM of nine chagasic IgG experiments in each groups. (B) Bar plot: anti-$M_2$ peptide chagasic IgG action on NOS activity. Rat atria were incubated during 2 h in absence (basal) or in presence of $1\times10^{-7}$ M chagasic IgG alone or in presence of different NOS isoform inhibitors at the same concentration used above. Values are mean±SEM of nine chagasic IgG experiments in each group. $*p<0.05$ versus chagasic IgG alone.

neutralized after pre-incubating the IgG with the $M_2$ synthetic peptide and by the $M_2$ selective mAChR antagonist AF-DX 116. Current analysis of the effects on PLC, PKC, calcium–calmodulin, and NOS inhibition showed that chagasic IgG–$M_2$ mAChR stimulation increases NO/cGMP accumulation as a result of activation of PLC, PKC, calcium–calmodulin and NOS activities.

### 3.5. Arachidonic acid cascade enzymes implicated in chagasic IgG generated $PGE_2$

In order to discern which arachidonic acid (AA) cascade enzymes are implicated in chagasic IgG-generate $PGE_2$ production, several inhibitors of this enzymatic cascade reaction were used. It can be seen in Fig. 6 that the inhibition of $PLA_2$ by OBAA ($5\times10^{-6}$ M) or COX-2 by DuP 697 ($5\times10^{-8}$ M) prevented the stimulatory action of chagasic IgG-induced $PGE_2$ production, while COX-1 inhibition by FR 122047 ($5\times10^{-8}$ M) had no effect. These results indicate that chagasic IgG–$M_2$ mAChR stimulation may trigger production of $PGE_2$ as a result of an increase in iNOS pathways with $PLA_2$ and COX-2 activation, ruling out COX-1 participation.

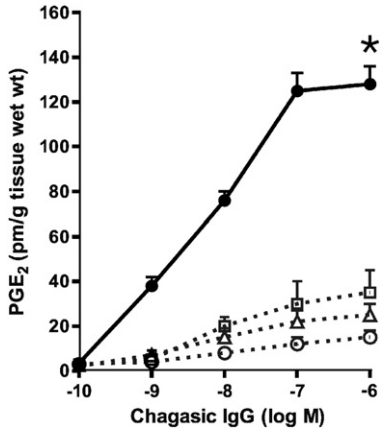


Fig. 5. Concentration–response curve of anti-$M_2$ peptide chagasic IgG (●) induced $PGE_2$ accumulation effect in absence (●), or in presence of $5\times10^{-6}$ M ODQ (○), $5\times10^{-9}$ M calphostin C (Δ) or $5\times10^{-6}$ M U-73122 (□). Values are mean±SEM of nine chagasic IgG experiments in each groups $*p<0.0001$ between chagasic IgG alone and the other groups.

Table 4
Effect of chagasic IgG upon enzymatic activities and PI turnover

| Additions | PI | PKC | cGMP | NOS |
|---|---|---|---|---|
| Basal | 192±12 | 1.4±0.1 | 42±3.2 | 260±18 |
| Chagasic IgG | 383±22* | 6.4±0.3* | 109±11* | 748±21* |
| Chagasic IgG+U-73122 | 178±15** | 1.3±0.4** | 45±5** | 250±15** |
| Chagasic IgG+TFP | 356±21 | 5.3±0.1 | 75±6** | 385±20** |
| Chagasic IgG +Calphostin | 422±35 | 1.6±0.1** | 43±4** | 233±20** |
| Chagasic IgG+l-NMMA | 356±25 | 5.5±0.2 | 40±5** | 219±15** |
| Chagasic IgG+$M_2$ peptide | 205±22** | 1.7±0.2** | 50±5** | 280±28** |
| Chagasic IgG+AF-DX 116 | 215±23** | 1.5±0.1** | 43±5** | 273±25** |
| Carbachol | 396±28* | 6.8±0.3* | 105±10* | 860±29* |
| Normal IgG | 213±21 | 1.6±0.2 | 48±4 | 270±28 |

Influence of enzymatic inhibitors.
Values are mean±SEM of five experiments in each group performed in duplicate. Enzyme activities were measured after incubation during 2 h rat atria in presence of anti-$M_2$ peptide IgG (Chagasic IgG $1\times10^{-7}$ M) or carbachol $1\times10^{-7}$ M or normal IgG $1\times10^{-7}$ M with or without enzyme inhibitors. U-73122 $5\times10^{-6}$ M, TFP $5\times10^{-6}$ M, calphostin $5\times10^{-9}$ M and L-NMMA $5\times10^{-5}$ M. Phosphoinositides (PI) were expressed in area/mg; membrane PKC in pmol/min/mg protein; NOS and cGMP in pmol/g tissue wet wt. Student' "*t*" test: $*p<0.001$ comparing with Chagasic IgG alone.

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### 3.6. Stimulation of NOS and COX mRNA gene expression by chagasic IgG

In order to define the role of different NOS isoforms due to chagasic IgG activation of $M_2$ mAChR, the iNOS, eNOS and nNOS gene expression was determined by RT-PCR amplification. Using specific primers for iNOS, eNOS and nNOS mRNAs, the product of RT-PCR amplification showed single clear bands of the predicted size (Fig. 7). Semi-quantitative RT-PCR analysis demonstrated that stimulation with anti-$M_2$ peptide IgG ($1\times10^{-7}$ M) for 2 h increased iNOS, nNOS, and eNOS mRNA levels in a concentration-dependent manner. In the case of iNOS, maximal expression was observed at $1\times10^{-8}$ M, while the levels of nNOS and eNOS increased with the IgG concentration raising their maximal expression at $1\times10^{-6}$ M. Moreover, the levels of iNOS mRNA obtained in the presence of chagasic IgG were significantly higher than those of the other NOS isoforms ($p<0.001$) at any studied concentration.

### 3.7. Selective COX-2 mRNA levels stimulated by chagasic IgG

To settle the role of COX isoforms in the action of chagasic IgG on atria $M_2$ mAChR activation, specific primers for COX-1 and COX-2 mRNA were used. Fig. 8 shows RT-PCR products and the semi-quantitative RT-PCR analysis demonstrating that anti-$M_2$ peptide IgG ($1\times10^{-7}$ M) stimulation for 2 h increased COX-2 mRNA levels (Fig. 8A), with no modificating of COX-1-mRNA levels (Fig. 8B). The fraction depleted of antipeptide chagasic IgG had no effect (data not shown). The same Fig. 8A shows that U-73122 , aminoguanidine and ODQ attenuate the stimulatory effect of anti-$M_2$ peptide IgG on COX-2 mRNA levels. These results together regarding mRNA expression demonstrate that chagasic IgG acts as an inducer of both iNOS and COX-2 mRNA. The same pathway that activates COX-2 expression activated iNOS expression (PLC , iNOS and guanylate cyclase).

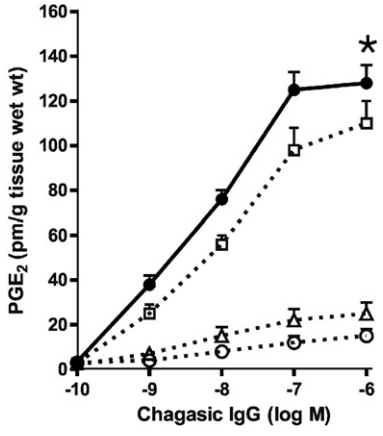


Fig. 6. Concentration–response curve of anti-$M_2$ peptide chagasic IgG-induced $PGE_2$ accumulation effect in absence (●), or in presence of $5\times10^{-6}$ M OBAA (○), $5\times10^{-8}$ M DuP 697 (Δ) or $5\times10^{-6}$ M FR 122047 (□). Values are mean±SEM of nine chagasic IgG experiments in each groups $*p<0.0001$ versus chagasic IgG alone and the IgG plus OBAA or DuP 697.

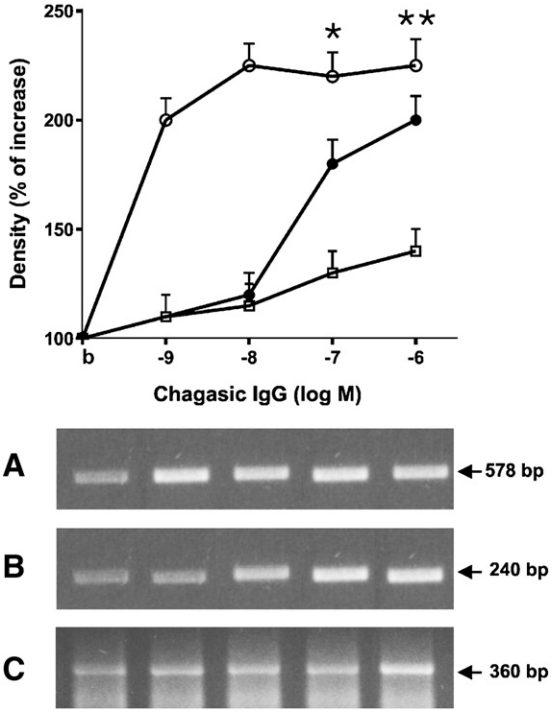


Fig. 7. Anti-$M_2$ peptide chagasic IgG action on semi-quantitative RT-PCR analysis for iNOS (○), eNOS (□) and nNOS (●) mRNA expression. Rat atria were incubated during 2 h in absence (basal) or in presence of different anti-$M_2$ peptide chagasic IgG concentrations. Values are mean±SEM of seven chagasic IgG experiments in each group RT-PCR products obtained from chagasic IgG dose–response curves on iNOS (A), nNOS (B) and eNOS (C) are shown. $*p<0.0001$ versus eNOS.

## 4. Discussion

Sera from chagasic dysautonomic patients contain high amounts of $PGE_2$, that correlate with the presence of serum autoantibodies. These autoantibodies recognize cardiac sarcolemma and are able to interact with the second extracellular loop of the human $M_2$ mAChR. The molecular interactions of chagasic antibodies with myocardial $M_2$ mAChR have the capacity to generate the proinflammatory substances $PGE_2$ and NO; acting as inducer of COX-2 and iNOS mRNA.

The finding that there is an association between functional active serum anti-$M_2$ mAChR peptide autoantibodies with the presence of signs of parasympathetic dysautonomia in asymptomatic chagasic patients allows us to suggest that these antibodies may serve as an early marker of Chagas' associated dysautonomia. Reduced vagal tonus was documented in chagasic patients with both normal ECG and left ventricular function [22]. The presence of autoantibodies in asymptomatic chagasic patients without

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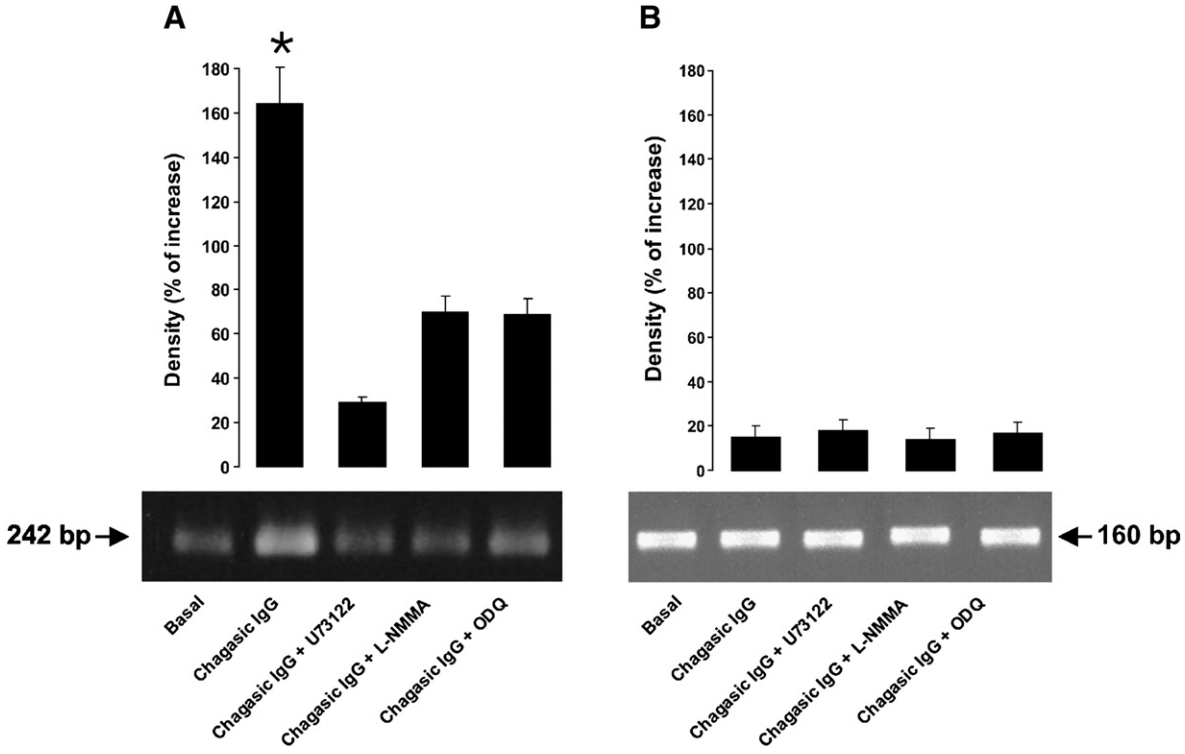


Fig. 8. Anti-$M_2$ peptide chagasic IgG action on semi-quantitative RT-PCR analysis for COX-2 (A) and COX-1 (B) mRNA expression. Rat atria were incubated during 2 h in absence (basal) or in presence of $1 \times 10^{-7}$ M anti-$M_2$ peptide chagasic IgG alone or incubated with U-73122, aminoguanidine and ODQ. Values are mean ± SEM of seven chagasic IgG experiments in each group RT-PCR products obtained from chagasic IgG dose–response curves on COX-2 (A) and COX-1 (B) are shown. $*p < 0.0001$ between chagasic IgG alone and the other groups.

dysautonomia (group II) might be explained by the fact, that during the course of the disease the peak serum concentration of $M_2$ mAChR antibody precedes the binding on cardiac mAChR, as described in chagasic myocarditis mice [15]. In previous work we showed that individuals with chronic chagasic cardiomyopathy had elevated levels of serum anti-$M_2$ mAChR autoantibodies. Moreover, we observed a significant correlation between the levels of serum $M_2$ mAChR autoantibodies and the chronotropic incompetence during exercise in chronic chagasic cardiomyopathy patients, despite the apparent lack of correlation with parameters of ventricular dysfunction [23]. Those and these data point to an important role of anti-$M_2$ mAChR autoantibodies in the pathogenesis of the dysautonomia. Prospective studies are needed to clarify whether or not the antibodies are predictive markers of Chagas' heart disease development.

The major new finding of this work was the demonstration that anti-$M_2$ mAChR antibodies behaving as cholinergic agonist, have the capacity to alter the rate of transcription of specific proinflammatory target genes, triggering the production of $PGE_2$ in response to receptor-mediated signaling events at the cell membrane. The transcription is rapidly induced following receptor activation and therefore, the target genes can be classified as early gene [20]. By the fact, we demonstrate a positive regulation of COX-2 and iNOS mRNA in the early stages, after the activation of cardiac $M_2$ mAChR by chagasic autoantibodies during 2 h. The expression of COX-2 and iNOS early genes, could play an important role in coupling receptor stimulation to long term tissue responses [24]. Therefore, the anti-cardiac mAChR autoantibodies might play a role in the pathophysiological mechanisms underlying the relevant inflammatory process described in chagasic myocardial disease.

A diagram to tight together the various systems studied and proposing a model by which anti-$M_2$ peptide IgG from chagasic patients might induce COX-2 and iNOS mRNA, triggering the production of proinflammatory mediators is shown in Fig. 9.

Increasing evidence suggests that there is considerable "cross talk" between NO and PGs biosynthesis, pathways involving an active back modulation operated by reaction end products [17]. However the final effect of these interactions depends on which circumstances they are produced. Under normal conditions the constitutive isoforms of NOS (eNOS and nNOS) and COX-1 are virtually in all organs and they help to maintain normal physiological functions, such as cytoprotection. In contrast, in inflammatory setting the inducible isoforms of these enzymes (iNOS and COX-2) are detected, resulting in the production of large amounts of proinflammatory and cytotoxic NO and PGs [25]. These proinflammatory agents account for acute and chronic inflammation, immunological alterations and cytotoxic tissue damage [17]. The proinflammatory mediator (COX-2) and its product ($PGE_2$) are induced in ischemic

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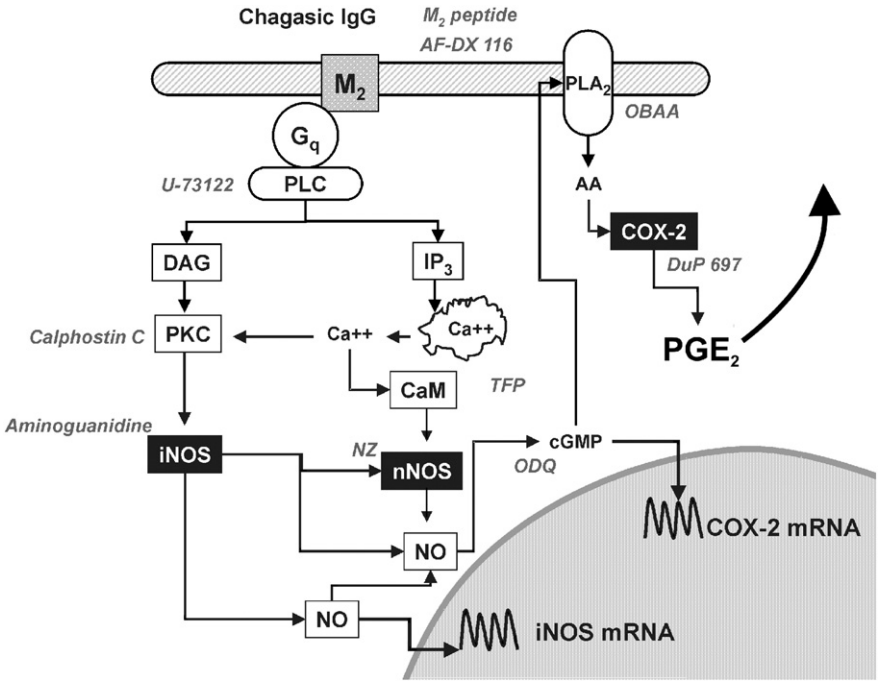


Fig. 9. Proposed model to explain the mechanism whereby chagasic IgG up-regulates iNOS and COX-2 mRNA levels and $PGE_2$ generation in isolated rat atria. Chagasic anti-$M_2$ peptide IgG acting on $G_q$-protein activates PLC, mediating production of inositol triphosphate ($IP_3$) and 1–2-diacylglycerol (DAG). $IP_3$ triggers intracellular release of calcium store ($Ca^{2+}$). Free $Ca^{2+}$ binds to calcium–calmodulin and the $Ca^{2+}$/calmodulin complex (CaM) activates nNOS with NO production. Alternatively, the rise in cytosolic $Ca^{2+}$ sensitizes PKC activation via DAG. Subsequent PKC translocates to the membrane, which in turn increases both iNOS activity and iNOS mRNA levels. On the other hand, the increase production of NO activates soluble guanylate cyclase with and increase of cGMP production. NO and cGMP activates phospholipase $A_2$ ($PLA_2$) with subsequent activation and induction of COX-2 mRNA levels, which induce generation of $PGE_2$. Inhibitory agents are indicated in italics.

heart and in heart failure contributing to inflammatory cell infiltration [26]. Accordingly, it has been shown that COX-2 is an immediate early response gene, that can be induced by hypoxia and by a variety of proinflammatory factors [27]. In this study we show that the production of $PGE_2$ by chagasic autoantibodies was mainly generated by COX-2 activation, as it was prevented by specific blockade of this enzyme. So, the inflammatory process described in chagasic myocardium might be attributed partly, to autoantibodies fixation on sarcolemma, that by interacting with $M_2$ mAChR, triggers the $PGE_2$ production via COX-2 activation and expression.

In our study, we provide evidence that the activation of COX-2 and the immediate production of COX-2 mRNA by chagasic autoantibodies was preceded by NOS activation, which in turn catalyzes $PLA_2$-AA release. No effect was observed regarding COX-1. Moreover, in this study we rise the possibility that COX-2 enzyme represents an important endogenous "receptor" target for different NOS isoform. The nNOS and iNOS are activated by chagasic autoantibody with major participation of iNOS. Thus, iNOS signaling system appears to be a key factor for chagasic IgG-induced $PGE_2$ generation, and iNOS products NO and cGMP are able to increase the rate of transcription of COX-2 in response to $M_2$ mAChR activation. COX-2 is known to be a co-inducer together with iNOS in cardiac myocytes, in response to stress [28,29]. In this sense, the signaling elements that control the expression of COX-2 during cardiac $M_2$ mAChR–antibody interaction are similar to those that control the induction of iNOS [20], since they include PLC, and PKC activation. The fact that the inhibition of soluble guanylatecyclase reduced COX-2 induction suggests the participation of cGMP in $PLA_2$-AA-COX-2 immediate early response resulting from antibody interaction with $M_2$ mAChR..

Myocarditis is associated with several well established autoimmune conditions [30]. Recognition of pathogens such as protozoa, by the innate immune system leads to release of proinflammatory cytokines, that both reduce infection and increase chronic inflammatory heart disease [15]. In previous work we demonstrated that, in *T. cruzi* infection, elevated $PGE_2$ levels are able to modulate tolerance to chronic Chagas' disease [31]. One of the major sources of $PGE_2$ are $CD8^+$ T cells [32], which release large amount of $PGE_2$ when they are activated by $M_2$ mAChR autoantibodies [33]. This antibody-host immune competent cell recognition modulates immune response allowing to parasite to survive, leading to disease chronicity [32,33]. The existence of nicotinic cholinergic anti-inflammatory and protective mechanism by which stimulation of the vagus nerve and cholinergic agonist inhibiting cytokine synthesis by macrophages has been described [34,35]. By the fact, this nicotinic-macrophage protection against cytokine-mediated disease, appears to be present in Chagas' disease since macrophages from mice acutely infected with *T. cruzi* were not able to increase $PGE_2$ generation like $CD8^+$ T cell did [31]. The muscarinic

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cholinergic activity is involved in survival to *T. cruzi* infection [15] because atropine treatment increased parasitemia and mortality infected mice [31]. Also, $PGE_2$ levels diminished by atropine [33] and COX inhibitors mimicked the effects of atropine. Beside this immunomodulatory action of the $M_2$ mAChR antibody, here we show another effect of the chagasic IgG, which by targeting cardiac $M_2$ mAChR, it acts as inducer of proinflammatory molecules gene expression. Thus, the increase production of proinflammatory agents by immune competent cells and myocardium triggered by the autoantibodies might modify two important mechanisms. On one hand, it might induce the immunosuppression response [33], and on the other, it might exacerbate or maintain the chronic inflammatory heart disease. In fact, the *in vivo* treatment of *T. cruzi* infected mice with the $M_2$ mAChR peptide prevents both cardiac dysfunction and reduce myocardial inflammatory foci and myocardial parasite nest. This effect was attributable to the capacity of peptide to impair the antibody fixation on myocardium and lymphocyte $M_2$ mAChR. The later effect avoided the capacity of *T. cruzi* to attach to the host cell, preventing invasion and intracellular multiplication [15].

In the current investigation serum anti-$M_2$ mAChR antibody significantly correlates with serum $PGE_2$ levels in chagasic patients with dysautonomia. Furthermore, serum anti-$M_2$ mAChR IgG induced $PGE_2$ production in myocardium. So, the similarities of these experimental and clinical studies raise a question: could there be a common explanation for the increased $PGE_2$ serum levels and "abnormal" parasympathetic function test of chagasic patients with dysautonomia. If this is a case, these results are consequence of an enhanced parasympathetic activity on the cardiovascular system of chagasic patients mediated by the cardiac muscarinic autoantibodies.

The fact that anti-$M_2$ mAChR antibody induces COX-2 and iNOS activity and expression tempts us to speculate that the antibody can have direct influence on cardiac remodeling through cytokine effects on fibrosis and cardiac hypertrophy [30], while regulating the degree of inflammation.

To our knowledge, there has been no previous report showing that an antibody interacting with heart mAChR can act as expression inducer of proinflammatory mediators.

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