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# Detection of mRNA encoding $H_1$ receptor and iNOS by RT-PCR in autoimmune myocarditis with special reference to changes in heart contractility

Nora Goren[a,b,c], Leonor Sterin-Borda[a,b,c], Ramón Bartrons[b], Ricardo Gomez[c], Enri Borda[a,c,*]

[a]*Pharmacologic Unit, School of Dentistry, University of Buenos Aires, Marcelo T. de Alvear 2142, 4 to Floor Sector B, 1122 Buenos Aires, Argentina*

[b]*Department of Physiological Sciences, University of Barcelona, Barcelona, Spain*

[c]*Consejo Nacional de Investigaciones Científicas y Técnicas de la República Argentina (CONICET), Argentina*

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**Abstract**

Cardiac tissue from autoimmune myocarditis mice was studied to evaluate the expression and biological activity of mRNA encoding $H_1$ receptor and iNOS. BALB/c inbred mice were immunized with heart protein and sacrificed at 20, 45 and 50 days post immunization. Heart contractility studies and RT-PCR assays were performed. Heart from autoimmune myocarditis mice show mRNA iNOS-related dysfunction with a decrease in heart contractility. This effect was accompanied with an increase production of cyclic GMP and was improved by treating autoimmune mice with an inhibitor of iNOS activity. In addition, autoimmune myocardium expressed an active histamine $H_1$ receptor mRNA coupled to phospholipase C. The activation of $H_1$ receptor by ThEA stimulated both phosphoinositide hydrolysis and heart contractility. Normal myocardium did not expressed neither iNOS mRNA nor $H_1$ receptor mRNA. In conclusions: the development of autoimmune cardiac dysfunction was associated with the expression of iNOS mRNA, cyclic GMP accumulation and the expression of an active histamine $H_1$ receptor mRNA with increase production of inositol phosphates. These protein emergence during the course of autoimmune myocarditis may be involved a distinct compensatory mechanism operating in this disease. 

*Keywords:* Autoimmunity; Heart; Contractility; Cyclic GMP; mRNA $H_1$ receptor; mRNA iNOS

## 1. Introduction

Experimental myocarditis models obtained by means of viral, parasitic or heart protein immunization closely mimic human myocarditis. A feature common to all of them is the presence of myocardial lesions, lymphomononuclear infiltrates in the heart associated with the induction of heart autoantibodies [1] and autoreactive T-cells [2]. Many of these events are associated with depression of heart function and are characterized by activation of the immune system with the release of soluble inflammatory factors, such as interferon gamma (IFN $\gamma$), interleukins and arachidonic acid metabolites which have been implicated in the pathogenesis of cardiac failure [1].

In this regard, in the myocardium nitric oxide (NO) release by cytokine-induced activation of the L-arginine nitric oxide cascade [3] is likely to be one of the cellular signalling events that could lead to cardiac contractile dysfunction [4]. A negative ino-

*Corresponding author. Tel.: +54-11-4964-1276; fax: +54-11-4508-3958.

*E-mail address:* enri@farmaco.odon.uba.ar (E. Borda).

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tropic effect of cytokines on heart mediated by NO has been described [5]. A significant activity of inducible nitric oxide synthase (iNOS) in heart tissue from patients with idiophatic dilated cardiomyopathy [6], in experimental autoimmune myocarditis rats [7] and viral myocarditis [8] has also been described.

On the other hand, it is well known that histamine is a mediator of the immediate hypersensitivity reaction of the heart and plays an important role as a modulator of immune response [9]. Moreover, the contribution of histamine to the functional crisis that characterizes the anaphylactic reaction of the heart has been documented. Also, during immunologic reaction endogenous histamine is released from the heart [10].

The heart exposed during the immune response to low concentrations of histamine showed modification in automaticity and contractility. Thus, we have previously shown that autoimmune myocarditis mice expressed histamine $H_1$ receptors that was absent in normal heart. Moreover, the stimulation of $H_1$ receptor by the agonist triggers positive inotropic effect in autoimmune atria [11].

The aim of the present investigation was two fold: firstly, to study the role of NO and cyclic GMP (cGMP) accumulation in the altered contractile behaviour of autoimmune heart and the possible modulation by the histamine $H_1$ receptors activation. Secondly, to show the presence of mRNA encoding $H_1$ receptor and iNOS in autoimmune heart by the use of reverse transcription (RT) of total mRNA and subsequent polymerase chain reaction (PCR).

## 2. Materials and methods

### *2.1. Animals and schedule of immunization*

BALB/c inbred male mice, 45 days old and weighing between 38-40 g, food and water ad libitum, were used in this study. They were separated into three groups of the same age and injected with myocardium plus complete Freund's adjuvant (group I), saline (group II) or with complete Freund's adjuvant (group III). Immunization procedures were carried out as reported previously [12]. Briefly, hearts were carefully removed and homogenized in phosphate buffered saline (PBS) at 4°C. Homogenate was passed through meshes and the protein concentration adjusted and emulsified with complete Freund's adjuvant (1:1). The mice were given subcutaneous injections into both sides of the dorsal region with 0.4 ml of the corresponding emulsion (day 1); a booster dose with similar characteristics was given 21 days later. Electrocardiograph studies mainly showed an enlargement of the QRS complex which appears as a greater lengthening of waves. Some immunized and non-immunized (control or normal) mice received in vivo treatment with 2-amino-4-methylpyridine 0.3 mg/kg/day s.c. for 3 days [13] before they were killed. As control, normal mice were injected with PBS, using the equivalent administration cited above. All studies were done following a double blinded experimental protocol. The investigation conforms with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1985).

### *2.2. RNA isolation: reverse transcription PCR (RT-PCR) amplification*

Total RNA was extracted from frozen whole heart from normal and autoimmune mice at 20, 45 and 50 days after the first injection, using guanidinium isothiocyanate method [14]. The RNA (5 μg sample) was reverse -transcribed using a Ready-To-Go T-Primed First-Strand Kit from Pharmacia Biotech (Upsala, Sweden) and N terminus of 240-bp iNOS and 1000-bp histamine $H_1$ receptor sequence was amplified by polymerase chain reaction (PCR). This nucleotide sequence is possess low homology to the constitutive type NOS sequence [15,16]. The nucleotide sequence of the 1000-bp fragment showed very high similarity with the histamine $H_1$ receptor sequences of bovine, rats and human suggesting that it was part of $H_1$ receptor gene [17]. High molecular weight genomic DNA was used as a template for the PCR analysis. PCR amplification was carried out using the reverse transcribed RNA with the iNOS up primer (5′-CACGAATTCGCTTGCCCCTGGA-3′) and iNOS down primer (5′-ATAG-GATCCTTTGATCCTCACA-3′) and histamine $H_1$ receptor up primer (5′-GCGAATTCGGAT-TATGTGGCCAGCAC-3′) and histamine $H_1$ receptor down primer (5′-CAGGATCCGTTGAT-GTAGCCCAGCCA-3′) and one unit of Taq DNA

polymerase; using a Gene Amp PCR System 2400 from Perkin Elmer (USA). The PCR conditions were 5 min at 94°C and followed by 35 cycles of 1 min at 94°C, 1 min at 56°C, 1 min at 72°C and 7 min at 72°C.

### 2.3. Mechanical activity

Mice were killed by decapitation. The atria from normal and autoimmune mice were separated from the ventricles and strip of the right ventricle was excised. Tissues were attached to a glass chamber and immersed in tissue bath containing a modified Krebs-Ringer bicarbonate (KRB) solution gassed with 5% $CO_2$ in oxygen at 30°C pH 7.4 as described previously [18]. A preload tension of 350 mg was applied to the tissues and they were allowed to stabilization for a period of 30 min. The initial control values for tension of isolated atria or ventricles were recorder using a force transducer coupled to an ink writing oscillograph. Atrial preparation was paced with a bipolar electrode using a SK4 Grass Stimulator. The stimuli had a duration of 2 ms and their voltage was 10% above threshold. Inotropic effect ($dF/dt$) were assessed by recording the maximum rate of isometric tension development during electrical stimulation at a frequency of 240 beats-per-minute. Right ventricle strips were expose to field stimulation with 1 ms duration at 50 beats/min of frequency. Results were expressed as gram-per-second (g/s).

### 2.4. Cyclic GMP assay

Atria or ventricles from normal and autoimmune mice were incubated in 1 ml of KRB with 1 mM of 3-isobutyl-1-methylxanthine and were gassed with carbogen and shaking at 37°C for 30 min. After incubation, preparations were homogenized in 2 ml of absolute ethanol and centrifuged at 6000 *g* for 15 min. Then, the pellets were rehomogenized in ethanol:water (2:1) and supernatants were collected and evaporated to dryness. Residues were resuspended in 5 mM Tris–HCl pH 7.4 containing 8 mM theophilline, 0.45 mM EDTA, 6 mM mercaptoethanol and 0.005 M of sodium acetate buffer pH 6.2. Determination of cGMP was done using a RIA procedure with a cGMP $^{125}$I-RIA KIT from Dupont/New England Nuclear (USA).

### 2.5. Measurement of total labelled inositol phosphates (IPs)

Atria or ventricles from normal and autoimmune mice were incubated for 120 min in 0.5 ml of KRB gassed with 5% $CO_2$ in oxygen with 1 μCi [*myo*-$^3$H]-inositol ([$^3$H]-MI) (sp. act. 15 Ci/mmol) from Dupont/New England Nuclear and LiCl (10 mM) was added for determination of inositol monophosphate accumulation according to the technique of Berridge et al. [19]. ThEA was added 30 min before the end of the incubation period and the blocker 30 min before the addition of ThEA. Water soluble IPs were extracted after 120 min incubation following the method of Berridge et al. [19]. Preparations were quickly washed with KRB and homogenized in 0.3 ml of KRB with 10 mM LiCl and 2 ml chloroform/methanol (1:2) to stop the reaction. Then, chloroform (0.62 ml) and water (1 ml) were added. Samples were centrifuged at 3000 *g* for 10 min and the aqueous phase of the supernatant (1–2 ml) was applied to a 0.7 ml column of Bio-Rad AG (formate form) 1×8 anion-exchange resin (100–200 mesh) suspended in 0.1 M formic acid which had been previously washed with 10 mM Tris formic pH 7.4. The resin was then washed with 20 volumes of 5 mM *myo*-inositol followed by six volumes of water and IPs were eluted with 1 M ammonium formate in 0.1 M formic acid. One ml fractions were recovered and radioactivity was determined by scintillation counting. Peak areas were determined by triangulation. Results corresponding to the second peak were expressed as absolute values of area units under the curve per milligram of wet weight tissue (area/mg) following the criteria of Simpson's equation [20]. In order to confirm the absence of [$^3$H]-MI in the eluted peaks of IPs, chromatography in silica gel 60 F254 sheets (Merck) was performed using propan-2-*ol*/6N $NH_4OH$ (14:5) as the developing solvent following the procedure of Hokin-Neaverson and Sadeghian [21].

### 2.6. Drugs

The following drugs were used: 2-thiazolyl-

ethylamine (ThEA) kindly provided by Smith Kline Beechman Pharmaceuticals Laboratory (USA), U-73122 from RBI/Sigma Company (USA) and 2-amino-4-methylpiridine from Tocris Chemicals (USA). All concentrations quoted in the text represent the final values in the bath solution.

### *2.7. Statistical analysis*

Statistical significance was stated by two tailed *t*-test for independent populations. Analysis of variance and Student–Newman–Keuls test was employed when multiple comparisons were necessary. Differences between means were considered significant if $P$ was equal or less than 0.05.

## 3. Results

Fig. 1 shows the expression of iNOS mRNA (panel A) and histamine $H_1$ mRNA receptor (panel B) in the autoimmune heart by RT-PCR analysis. The expression of iNOS mRNA was first detected in day 20 and was continued and even more detectable in day 50 post immunization (panel A). The products of the expected size comparing with positive control corresponding to mRNA histamine $H_1$ receptor by agarose gel is also shown (panel B). There was no expression of iNOS mRNA and histamine $H_1$ receptor mRNA in the heart of normal mice.

To analyse if the expression of iNOS and histamine $H_1$ mRNA are involved in the functional behaviour of mice autoimmune heart, atria and ventricles contractility were studied. Fig. 2A (upper and lower panel) shows that basal contractility of autoimmune atria or ventricles decreased in function of time post isolation while no changes in normal preparations were observed. It is noted that $dF/dt$ initial values (zero time) is lower in autoimmune than in normal preparations. In vivo treatment with specific iNOS inhibitor (2-amino-4-methylpiridine) significantly improved the contractile activity of autoimmune atria or ventricles without changes in normal ones (data not shown).

In order to know if histamine $H_1$ receptors on autoimmune preparations are in active state, dose response curves of specific $H_1$ agonist ThEA on $dF/dt$ from autoimmune atria and ventricles were studied. It can be seen in Fig. 2B (upper and lower panel) that the addition of ThEA caused a dose dependent increment in $dF/dt$ on isolated autoimmune preparations without any effect on normal ones.

As it is know that histamine $H_1$ receptor activity is regulated by phospholipase C (PLC), inhibitor of this enzyme was used. The incubation of autoimmune atria or ventricles with U-73122 ($5\times10^{-6}$ M) attenuated the inotropic positive action of ThEA. On normal preparations the PLC inhibitor was without effect (data not shown).

All of these results point to the participation of iNOS in the decrement of basal contractility observed in autoimmune heart and the presence of active histamine $H_1$ receptor able to improve the autoimmune myocardial contractility.

Some of the physiological action of NO on heart are mediated by the activation of guanylate cyclase by increasing production of cGMP. Thus, we investigated if the decrement in basal contractility of autoimmune heart was accompanied by modification

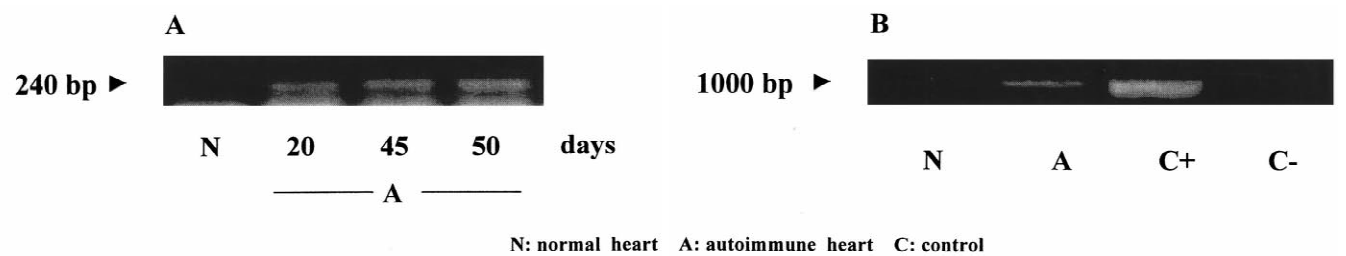


Fig. 1. Expression of iNOS mRNA (Panel A) and histamine $H_1$ mRNA (Panel B) in autoimmune heart (A) by RT-PCR analysis. No expression of iNOS mRNA and $H_1$ mRNA was observed in normal heart (N). Positive control ($C^+$) and negative control ($C^-$) corresponding to mRNA $H_1$ receptor by agarosa gel, are also shown.

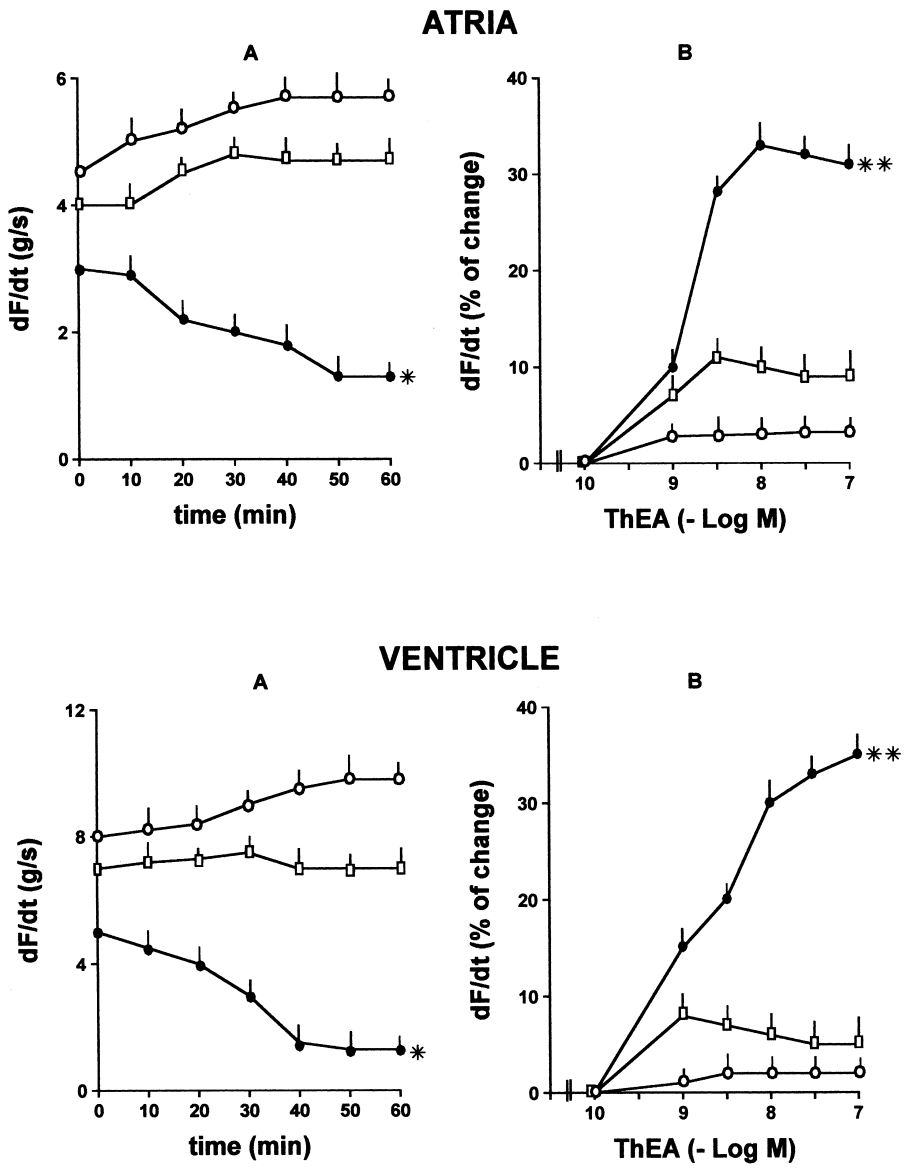


Fig. 2. Atria and ventricles contractility. Panel A: contractility of autoimmune (●) and normal (○) atria or ventricle in function of time post isolation. Effect of in vivo treatment with 2-amino-4-methylpyridine on autoimmune preparations (□). Panel B: dose–response curve of ThEA on autoimmune (●) and normal (○) atria or ventricles. Effect of U-73122 on ThEA action upon autoimmune atria or ventricles (■) is also shown. Results are mean±S.E.M. of seven mice in each group. *Differ significantly from autoimmune atria treated with 2-amino-4-methylpyridine with $P<0.001$. **Differ significantly from autoimmune atria treated with U-73122 with $P<0.001$.

in the production of cGMP. As can be seen in Fig. 3 the production of endogenous cGMP was significantly higher in autoimmune than in normal heart. The in vivo treatment with the specific iNOS inhibitor decreased the cGMP accumulation, raising similar levels than that observed in normal heart.

To confirm if phosphoinositide turnover is activated by the stimulation of histamine $H_1$ receptor, we measured the inositol phosphates formation in the presence of ThEA in both autoimmune and normal heart. Table 1 shows that ThEA increased IPs formation in myocardium from autoimmune preparations without effect in normal ones. The increment of IPs formation by ThEA was blocked by U-73122 (5×

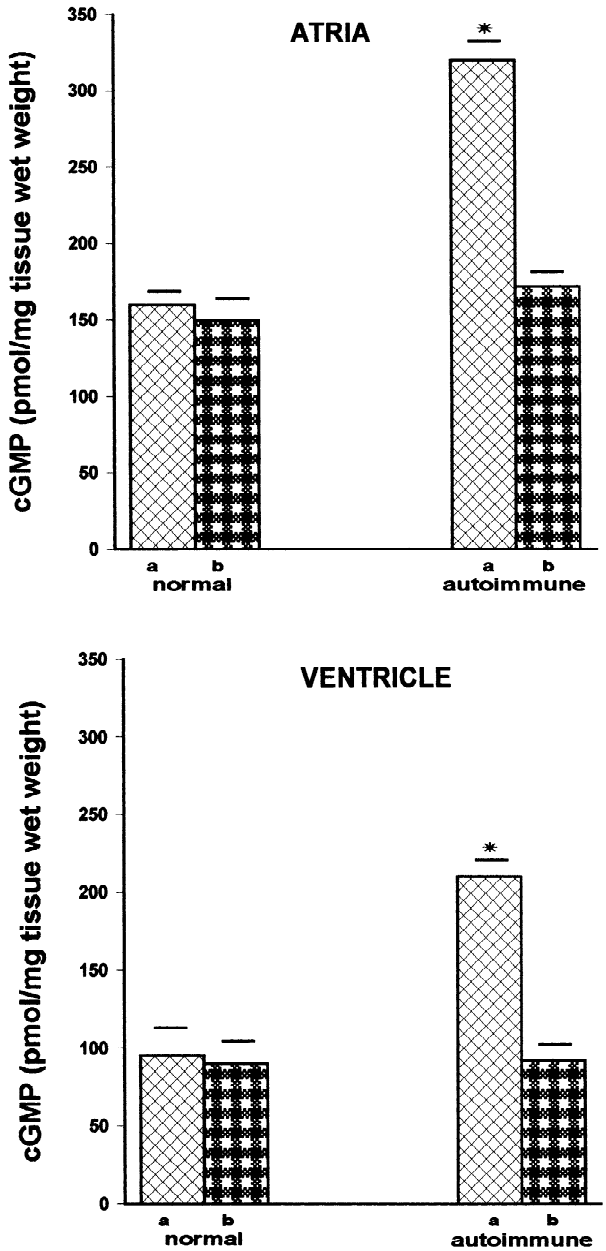


Fig. 3. Basal cyclic GMP (cGMP) on normal and autoimmune atria or ventricles was measured in homogenized tissue as described in Methods: (a) values without treatment; and (b) values obtained after the in vivo treatment with 2-amino-4-methylpyridine. Values are mean±S.E.M. of six mice in each group performed in duplicate. *Significantly different from other groups with $P<0.001$.

$10^{-6}$ M); pointing to the participation of PLC in both functional study (contractility) and signal transduction (IPs) triggered by histamine $H_1$ receptor activation.

Table 1
Influence of ThEA upon phosphoinositide (PI) turnover in autoimmune and normal heart[a]

| Addition | PI (area units/mg tissue wet weight) | | | |
|---|---|---|---|---|
| | Atria | | Ventricle | |
| | Autoimmune | Normal | Autoimmune | Normal |
| None | 212±14 | 196±12 | 180±13 | 160±10 |
| ThEA | 498±24* | 214±19 | 312±20* | 172±14 |
| U-73122+ThEA | 222±18 | 218±15 | 192±12 | 165±13 |

[a] Results correspond to the second peak. Values are mean±S.E.M. of seven experiments in each group. *Significantly different from none or U-73122+ThEA with $P<0.001$.

## 4. Discussion

The present work shows significantly iNOS and $H_1$ protein expression in autoimmune myocardium as shown by in vitro contractile study and RT-PCR assays, that was normally absent.

Here we examined the role of iNOS mRNA and histamine $H_1$ mRNA on experimental myocarditis induced by heart protein immunization that resemble human myocarditis [12,22]. We show that both iNOS and $H_1$ protein expression are involved in the alteration of autoimmune myocardium contractile behaviour. Results point to that iNOS-cGMP participate in the decrement of basal contractility of autoimmune heart while the presence of active histamine $H_1$, is perhaps a regulatory contractile factor that improved the contractile behaviour of the heart. This conclusion is based on the following observations: (1) using reverse transcription of total RNA and subsequent PCR we demonstrate the presence of mRNA encoding iNOS, that is accompanied with the decrease in contractility on autoimmune myocardium and increase production of cGMP. The inhibition of iNOS by in vivo treatment with a specific blocker, significantly improved the autoimmune myocardial contractility and decreased the accumulation of cGMP; and (2) the presence of mRNA encoding histamine $H_1$ receptor in myocardium appear to be in an active state, as the specific histamine $H_1$ agonist (ThEA) was able to increase autoimmune heart contractility. These effects appear to be related with the activation of phosphoinositide turnover via PLC, as both, contractility and inositol phosphates accumulation were blunted by PLC specific inhibitor agent.

Histamine $H_1$ receptor stimulation has been shown to stimulate the hydrolysis of phosphoinositides in guinea pig atria [23]. However, the role of inositol turnover in the regulation of cardiac muscle function is still unclear. In autoimmune heart our finding shows that the phosphoinositide hydrolysis subserves the $H_1$ receptor-mediated mechanical effect of ThEA, suggesting that this mechanisms could play an important role in autoimmune heart.

Previous studies demonstrated that autoimmune myocardium displayed two populations of $^3$H-mepyramine binding sites with low and high affinity for the histamine antagonist. The high affinity component seemed to be $H_1$ selective and was similar in both quality and number to that reported in one of the models of cardiac anaphylaxis [11]. There are study showing mRNA encoding histamine $H_1$ receptor coupled to the production of NO in human cranial arteries [24].

Endogenous NO is a potent vasodilating substance and has been suggested as a factor which participate in the pathogenesis of cardiac anaphylaxia [25]. Moreover, the iNOS has been implicated in maintenance of autoimmune heart disease [7]. Endogenously formed NO has been proposed to play a protective role during cardiac anaphylaxis, antagonizing the vasoconstrictor effect of released eicosanoids [26] and cytokines [27] that lead to an altered contractile function [28–30]. However, immunological generated NO, in addition to being cytotoxic for invading antigens, may also have similar adverse effects on host cells. So, organs in which the induction of iNOS occurs, showed signs of NO-dependent toxicity [7,31] that might lead to cell dysfunction and tissue damage.

In conclusion, we demonstrated in this study the expression of iNOS mRNA and histamine $H_1$ mRNA in autoimmune heart that are not present in normal heart. The histamine $H_1$ receptor stimulation increased contractility while the presence of iNOS is associated with cardiac contractile inhibition. These proteins emergence during the course of autoimmune myocarditis could involve a distinct compensatory mechanism operating in this disease.

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