Differential β_1 Cardiac Adrenoceptor Modulation of Nitric Oxide Isoforms by β_1 IgG from Patients with Periodontitis during Short-Term Hypoxia

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Abstract

Background: We demonstrated previously that serum IgG from periodontitis patients interacting with the second extracellular loop of the human cardiac β_1 adrenoreceptors triggers the production of second messengers. In this paper we quantified the production of nitrates/nitrites and nitric oxide (NO), which in turn induces nitric oxide synthase (NOS) mRNA expression. Methods: We determined using β_1 IgG, NOS activity isoforms, NOS expression, nitrate/nitrite assay, cGMP accumulation and PKC activity. Results: We established that serum β_1 IgG autoantibodies and NO might be considered as early markers in normoxia/hypoxia system in rat isolated atria. The β_1 IgG autoantibodies from periodontitis patients, while stimulating myocardial atria β_1 adrenoreceptors, exert an increase on NO levels indirectly quantified as nitrite/nitrate, which acts as NO-storage molecules with significant increase in neuronal NOS (nNOS) and inducible NOS (iNOS) mRNA levels in hypoxia conditions. The significant increase in nNOS/iNOS mRNA and NOS activity as well as in NO levels after short-time hypoxia in rat isolated atria was detected. The expression of these genes are related with the increase in atria dF/dt, cyclic GMP (cGMP) and protein kinase C (PKC) activity and resemble the results obtained by Isoproterenol, an β_1 adrenoreceptor agonist. Conclusion: These findings indicate that short-term hypoxia up-regulated rat atria NO/NOS system in the presence of β_1 IgG autoantibodies shows that an antibody interacting with rat atria β_1 adrenoreceptor can act as expression inducer of proinflammatory NO and its metabolites and that it might be useful and helps to maintain heart function and to prevent necrosis and subsequent loss of heart function during hypoxia.

Keywords

1. Introduction

Hypoxia is one of the most frequently encountered types of stress in health and disease. The heart suffers hypoxia in many circumstances, including ischemia, haemorrhage, pulmonary diseases, cardiac arrest and other cardiovascular problems. It has been suggested that nitric oxide (NO) plays a critical role in modulating cardiac function during hypoxia [1].

Nitric oxide is known to act as an intracellular mediator that can be considered a doubled-edged sword: low concentrations can exert beneficial effects, but high concentrations, if they persist uncontrolled, can be detrimental, with the generation of highly toxic compounds [2]. Three nitric oxide synthases (NOS) isoforms have been classically identified [2]: endothelial NOS (eNOS), inducible NOS (iNOS) and neuronal NOS (nNOS). Whereas eNOS and nNOS are usually constitutively expressed under physiological conditions, iNOS can be induced by certain stimuli, including hypoxia, antibodies, and cytokines [3]-[6]. Most of the NOS activity in the heart corresponds to eNOS, which is located in the vascular endothelium within the myocardium, as well as in the cardiomyocytes [5]. By contrast, nNOS appears much less prominent, being located in scattered nerves and ganglion cells [7]. Meanwhile, iNOS can be induced in macrophages, endothelial cells and cardiomyocytes [5] [7]-[9] in response to some stimuli, such as hypoxia [9]. However, the study of the cardiac NO/NOS system under normoxia and hypoxia conditions has been explored in the heart [10], but in the presence of beta adrenergic autoantibodies results are still controversial. On the other hand, NO plays a key role in the pathology and physiology of chronic inflammation [11] and in the neurodegenerative process [12].

Autoantibodies with functional activities against β_1-adrenoceptors (β_1 IgG) were described for the first time in patients with cardiomyopathy [13] [14], followed by the description of such β_1-AR antibodies in patients with dilated cardiomyopathy [15] [16]. Recent studies [17] suggest the involvement of serum β_1-IgG directed toward extracellular matrix components in the pathogenesis of certain types of periodontal disease. Initial studies of autoimmunity in the pathogenesis of periodontitis focused on detecting autoantibodies directed toward various self-antigens, such as autoantibodies against the human gingival fibroblast β_1 adrenergic receptors [18]. Thus, antihuman gingival fibroblast antibodies concomitant with antibacterial antibodies may contribute to the pathogenesis of periodontitis.

Periodontal disease is multifactorial infection [19] [20], and is caused by dental plaque biofilm, but the host inflammatory immune response modifies disease outcome [21]. Also, stress and local stimulation of the autonomic adrenergic system are cofactors that could contribute to the prevalence of disease and to disease progression [22].

We are now able to show that, in certain cardiovascular diseases and in periodontal disease, the second extracellular loop of the β_1-AR is the main antigenic domain recognized by the anti-β_1 AR autoantibodies present in the serum of periodontitis patients [23] [24], and it could also be a target for autoantibodies with functional activities [25].

On the other hand, periodontitis has been linked to systemic illnesses such as cardiovascular disease and stroke. Increasing evidence indicates that periodontal disease is a risk factor in coronary disease (ischemia) through endothelial cell dysfunction induced by periodontopathic bacteria, their products, or inflammatory mediators derived from infected periodontal tissue [26] [27].

Taking these observations jointly, we focused our research on the possibility that, in addition to the direct action of cardiac contractility, the β_1 IgG might contribute to the cardiac inflammatory reaction that characterized chronic periodontal illness [28]. We investigated whether the β_1 IgG can induce NOS activation and an increment of NO in the myocardium of rat atria during hypoxia, pointing to a novel insight into the mechanisms involved in the ability of β_1 IgG to act as an early inducer of myocardial protection during hypoxia. Moreover, this study provides evidences that β_1 IgG present in the serum of chronic periodontitis may serve as an early element of myocardial protection in acute hypoxia.

2. Materials and Methods

2.1. Patients

The study group consisted of 14 male adult patients with periodontitis (group I) who were attending the periodontology Clinic from the metropolitan area of Buenos Aires. The mean age was 41 years, with a range of 32 - 50 years. Healthy subjects (group II) were used as controls (12 male subjects) with a mean age of 38 years and a range of 30 - 46 years. The assessment of clinical parameters was carried out by a calibrated periodontist fol-

lowing the criteria on the basis of clinical parameters and the severity and the periodontal tissue destruction [29]. The characteristic clinical signs of periodontitis included the following: loss of clinical attachment; horizontal or/and angular alveolar bone loss; periodontal pocket formation; and gingival inflammation. To be included in the study, at least six sites with ongoing periodontal disease were required. Clinical measurements on patients with periodontitis included sites with alveolar bone loss > 2 mm and a pocket depth > 5 mm with bleeding and attachment loss > 3 mm. In the healthy subjects (control group), the probing depth was <3 mm and the attachment loss was <2 mm. Moreover, probing pocket depth and clinical attachment level were assessed at six sites per tooth and bleeding on probing at four sites per tooth. None of the subjects (patients in group I and controls in group II) had systemic illnesses and they were never-smokers. The patients with periodontitis had not received periodontal treatment or antibiotics within the preceding 5 months or any anti-inflammatory drugs 3 weeks prior to the study. Additionally, pocket probing depth (PPD: $\geq$5 mm), clinical attachment loss (CAL: $\geq$4 mm) and bleeding on probing: positive, are also determined. All of the patients consented to participate in the study and the investigation was conducted according to the tenets of the Declaration of Helsinki of 1975 as revised in 2000.

2.2. Human Sera and IgG Purification

Sera and the corresponding IgG were obtained from patients with chronic periodontitis and normal individuals. To obtain the sera, 6 ml of blood was obtained by venipuncture, allowed to clot at room temperature, and was separated by centrifugation at 2000 g and stored at −20˚C until used in assays. The IgG was obtained by precipitation with ammonium sulphate at 50%, followed by 3 washes and re-precipitation with 33% ammonium sulphate. More details in the determination of IgG were previously reported [18]. Briefly, the final concentration of IgG was 10 mg protein/ml.

2.3. Animals

Adult male Wistar strain rats (250 - 300 g) were used. The animals were housed in standard environmental conditions and fed with a commercial pellet diet and water ad libitum. The experimental protocol followed the Guide to The Care and Use of Experimental Animals (DHEW Publication, NIH 80-23). Rats were anesthetized with a mixture of ketamine and xylazine (50 and 5 mg·kg^{-1} respectively) and killed by decapitation. After rats were decapitated, atria were removed quickly and placed in a glass chamber containing Krebs Ringer bicarbonate (KRB) solution (pH 7.4), that was gassed with 5% CO_2 in oxygen at 37˚C.

2.4. Contractile Study

Rats were decapitated and atria were removed quickly and placed in a glass chamber containing Krebs Ringer bicarbonate (KRB) solution (pH 7.4), that was gassed with 5% CO_2 in oxygen at 37˚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 [24]. Then, the preparations were paced by means of a bipolar electrode using a SK4 Grass stimulator, with stimuli duration of 2 msec and a voltage that was 10% above threshold. The constant resting tension applied to the atria (preload tension) was 750 mg. The contractility (dF/dt) was assessed by recording the maximum rate of isometric force development above the externally applied resting tension. To obtain the maximum IgG effect, different concentrations of IgG were added to normal rat atria every 10 min. Control values (equal to 100%) referred to the dF/dt before the addition of different IgG concentrations.

2.5. Experimental Protocol

The isolated atria underwent a 50 min stabilization period under basal conditions, during which they were equilibrated in buffer gassed with 95% O_2, 5% CO_2 (37˚C, pH 7.4; pO_2 > 600 mmHg); followed by 50 min of hypoxia when the atria were equilibrated in buffer gassed with 95% N_2, 5% CO_2 (37˚C, pH 7.4; pO_2 > 100 mmHg). The adrenergic agonist (isoproterenol, 1×10^{-7} M) and the antibody (β_1 IgG 1×10^{-8} M) were added in the last 10 min before beginning the hypoxic period. The total duration time of this experimental design was 120 min and at the end of this period atria samples were fixed in 10% formaldehyde buffer and processed for biochemical analysis of NOS, cyclic GMP production, PKC determination and nitrites/nitrates determinations. All this

protocol was done in atria subjected to normoxia and hypoxia, as described above. In the blocking experiments, Atenolol (1×10^{-7} M, a β_1-specific adrenergic antagonist) and synthetic β_1-adrenergic peptide (5×10^{-5} M) were used. Normal β_1 IgG was used as control (basal values). All inhibitors were added at the beginning of the stabilization period (0 min).

2.6. Determination of NOS Activity

NOS activity was measured in rat atria tissue by the production of [U-14C]-citrulline from [U-14C]-arginine, as previously described [30]. Briefly, pulp was incubated for 30 min in 500 µl KRB solution that contained 18.5 kBq of L-[U-14C]-arginine. Inhibitors were added from the beginning of the incubation period, at the final concentrations indicated in the text, and the agonist Isoproterenol (1×10^{-7} M) and β_1 IgG (1×10^{-8} M) at 10 min before the end of incubation. Incubation was carried out in a 5% CO^2 in oxygen atmosphere at 37°C. Tissues were then homogenized in an Ultra Turrax homogenizer in 500 µl of medium that container 20 mmol·L^{-1} HEPES pH 7.4, 0.5 mmol·L^{-1} EGTA, 0.5 mmol·L^{-1} EDTA, 1 mmol·L^{-1} dithiothreitol, 1 mmol·L^{-1} leupeptin and 0.2 mmol·L^{-1} phenylmeth-ylsulphonyl fluoride at 4°C. Supernatants were applied to 2ml columns of Dowex AG 50WX-8 (sodium form), and [14C]-citrulline was eluted with 3 ml water and quantified by liquid scintillation counting (Beckman LS 6500, New York, NY, USA).

2.7. mRNA Isolation and cDNA Synthesis

Total RNA was extracted from rat atria tissue by homogenization using the guanidinium isothiocyanate method as previously described [31]: a 20 µl reaction mixture that contained 2 ng mRNA, 20 U RNase inhibitor, 1 mmol·L^{-1} dNTPs and 50 U Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI, USA). First-strand cDNA was synthesized at 37°C for 60 min.

2.8. Polymerase Chain Reaction (PCR) Procedures

NOS isoform mRNA levels were determined in both experimental conditions of normoxia and hypoxia as published elsewhere [11] [31]. Briefly, after initial denaturation at 94°C for 2 min, the cycle conditions were 45 cycles of 30 s denaturation at 94°C, 30 s annealing at 60°C and 45 s for enzymatic primer extension at 72°C for NOS isoforms. Different NOS isoforms mRNA levels were normalized with the levels of g3pdh mRNA present in each sample, which served to control for variations in RNA purification and cDNA synthesis [31].

2.9. Nitrite/Nitrate Analysis

After atria was incubated in KRB (500 µl) for 30 min with or without drugs (following the protocol used for NOS activity assays) during normoxia and hypoxia conditions, nitrite/nitrate levels were measured in tissue homogenates with a commercial kit (Caiman Chemical Laboratories, Am Arbor, MI, USA). Briefly, nitrate was converted to nitrite by incubation with nitrate reductase in the presence of nicotinamide adenine dinucleotide phosphate. Lactate dehydrogenase was then used to destroy excess NADPH. Equal volumes of sample and Griess reagent were incubated at room temperature. After 10 min, absorbance was read at 550 nm. The nitrite/nitrate concentration was determined by using sodium nitrate as a standard and the values were expressed as micro moles per milliliters (µM/ml).

2.10. Assay for Cyclic GMP (cGMP)

Rat atria (10 mg) were incubated in 1 ml KRB for 30 min and the ISO and β_1 IgG added in the last 15 min in normoxia and hypoxia. When blockers were used, they were added 25 min before the addition of the antibody and the drug. After incubation, atria was homogenized in 2 ml of absolute ethanol and centrifuged at $6000 \times g$ for 15 min at 4°C. Pellets were then re-homogenized in ethanol-water (2:1). Thereafter, to determine cyclic GMP production, the cyclic GMP 125 I-RIA KIT from Dupont New England Nuclear was employed. The cGMP results were expressed as in picomole/gram of tissue wet weight (pmol/g tissue wet wt).

2.11. Protein Kinase C (PKC) Activity Assay

Atria were incubated alone or in the presence of β_1 IgG, stimulant plus blockers or blockers alone for a total in-

cubation time of 60 min in KRB solution at 30°C and were frozen immediately in liquid nitrogen. PKC activity was purified from sub-cellular 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 sub-cellular cardiac fractions, following the instructions of the PKC assay system of Life Technologies. PKC specificity was confirmed by means of the PKC pseudo-substrate inhibitor peptide PKC provide by Gibco (Calbad, CA, USA) [32]. The data were expressed in picomoles of phosphate incorporated into the substrate per minute and per milligram of protein (pmol/min/mg protein).

2.12. Drugs

Isoproterenol (ISO) and atenolol were purchased from Sigma Chemical Co. (Saint Louis, MO, USA); L-NIO dihydrochloride (eNOS inhibitor), N-propyl-L-arginine hydrochloride (NZ) (nNOS inhibitor) and (S)-methylisothiourea sulphate (methylurea) (iNOS inhibitor) were from Tocris Cookson Inc. (Ellisville, MO, USA). Stock solutions were freshly prepared in the corresponding buffer.

2.13. Statistical Analysis

Student's t-test for unpaired values was used to determine the levels of significance. Analysis of variance (ANOVA) and the Student-Newman-Keuls test were employed when pair-wise multiple comparison procedures were necessary. Differences between mean values were considered significant at $P < 0.05$.

3. Results

3.1. Influence of β_1 IgG on Cardiac NOS Activity

Knowing that the amino acid sequence of rat and human β_1 cardiac adrenergic receptors-mediated effect of autoantibodies from periodontal patients on rat atria myocardium, using β_1 IgG from serum of periodontitis patients. **Figure 1** show in normoxia (upper panel) and hypoxia (lower panel) the ability of β_1 IgG to stimulate ni-
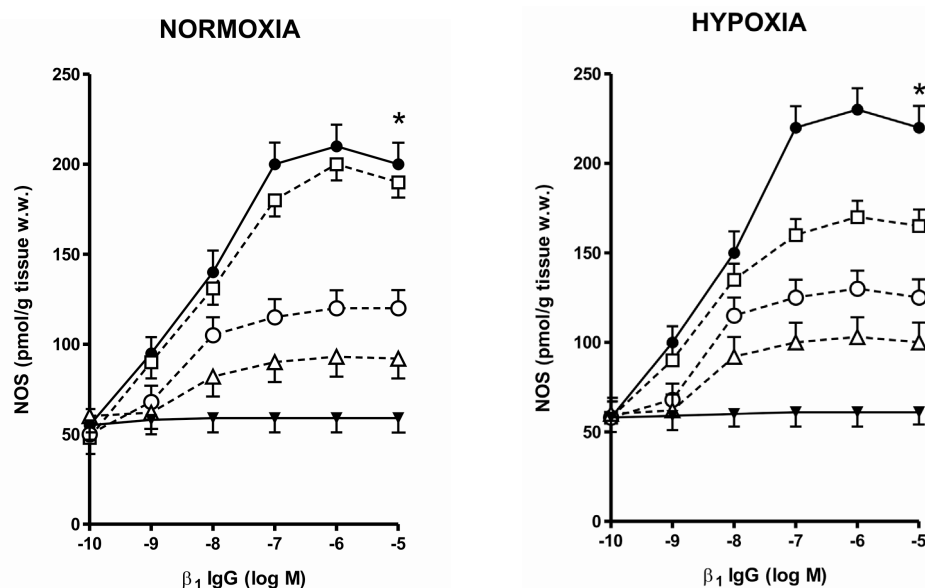

Figure 1. Dose-response curve of β_1 IgG from serum of periodontitis patients alone (●) and β_1 IgG from normal individuals alone (▼) or in the presence of L-NIO dihydrochloride (eNOS inhibitor) 5×10^{-6} M (Δ), N-propyl-L-arginine hydrochloride (NZ) (nNOS inhibitor) 5×10^{-6} M (○) and (S)-methylisothiourea sulphate (methylurea) (iNOS inhibitor) 1×10^{-5} M (□). Cardiac atria tissues were incubated with each concentration of the antibody for 15 min and inhibitors were added in the beginning of the experiments during 20 min before the antibody was added and NOS activity were assayed as described in materials and methods during normoxia and hypoxia respectively. Values are mean ± SEM of 7 experiments in each case performed by duplicate using a pooled of β_1 IgG from 14 periodontitis patients and 12 normal individuals.

tric oxide synthases (NOS) activity in a concentration-dependent manner. The β_1 IgG from normal individuals gave negative results.

To demonstrate which isoforms of NOS might be increased in atria during normoxia and/or hypoxia, isolated rat atria tissue was incubated with specific inhibitors of NOS isoforms. As can be seen in **Figure 1** upper panel and **Figure 1** lower panel, that the inhibition of eNOS and nNOS by NZ and L-NIO significantly decreased (P < 0.001) the NOS activity. In contrast, the inhibition of iNOS by methyl urea had no effect on iNOS activity in normoxia conditions (upper panel) but the iNOS activity in hypoxia conditions was significantly decreased (P < 0.001) (lower panel). All inhibitors had no effect on NOS activity in control experiments using normal β_1 IgG (data not show).

This observation strengthened the causal relationship between the increase in iNOS activity in the course of hypoxic conditions and these results confirm of whether high nitric oxide (NO) levels were involved in the increase activity of iNOS in rat atria myocardium during hypoxia conditions.

3.2. Stimulation of Cardiac NOS mRNA Gene Expression by β_1 IgG

Semi-quantitative RT-PCR analysis demonstrated that stimulation with β_1 IgG (1×10^{-8} M) for 2 hours, increased nNOS and eNOS in normoxia and hypoxia but only in hypoxia condition, iNOS isoforms increased by the action of this autoantibodies (β_1 IgG). Moreover, the iNOS mRNA obtained in the presence of β_1 IgG were significantly higher than those of the other isoforms (P < 0.001) in hypoxia conditions (**Figure 2**) at any studied concentration.

3.3. Biological Effect and Enzymatic Assay of β_1 IgG upon Rat Atria

This observation could have a causal relationship between the increase in iNOS activity in hypoxia and the presence of β_1 IgG. So, the increases in the concentration of total nitrite/nitrate in the atrium in normoxia, would correspond to an increase of nitric oxide caused by the activities of two isoforms namely, nNOS/eNOS while during the hypoxia iNOS activity would appear together with nNOS/eNOS as provocative element of increased of NO handset (**Figure 3**).

This would allow that the production ofNO in normoxia would correspond to 70% and 32% of the activity of nNOS and eNOS respectively. Furthermore, in hypoxia, production of NO come from the activity 28% of iNOS in a 28%, 40% of nNOS and 32% eNOS in our experimental conditions (**Figure 4**).
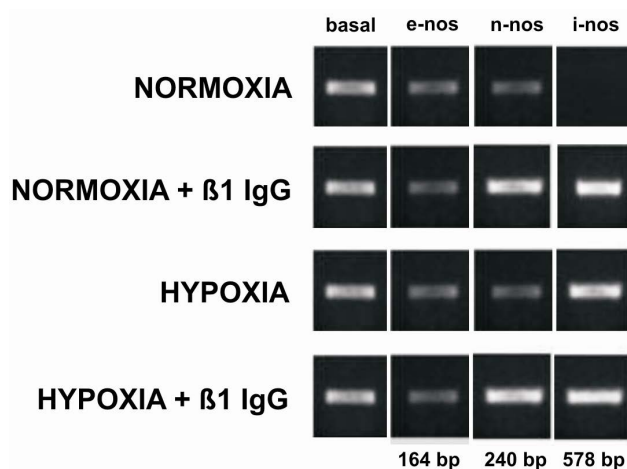

Figure 2. RT-PCR products for NOS isoforms: eNOS, n-NOS and i-NOS alone in normoxia (A) or in the presence of 1×10^{-8} M β_1 IgG in normoxia (B) or e-NOS, n-NOS and i-NOS in hypoxia alone (C) or in the presence of 1×10^{-8} M β_1 IgG in hypoxia (D). Rat atria myocardium were incubated for 2 hours in KRB solution under identical experimental conditions as those described for NOS activity. Reaction products shown are one representative from 6 atria tested in each group.
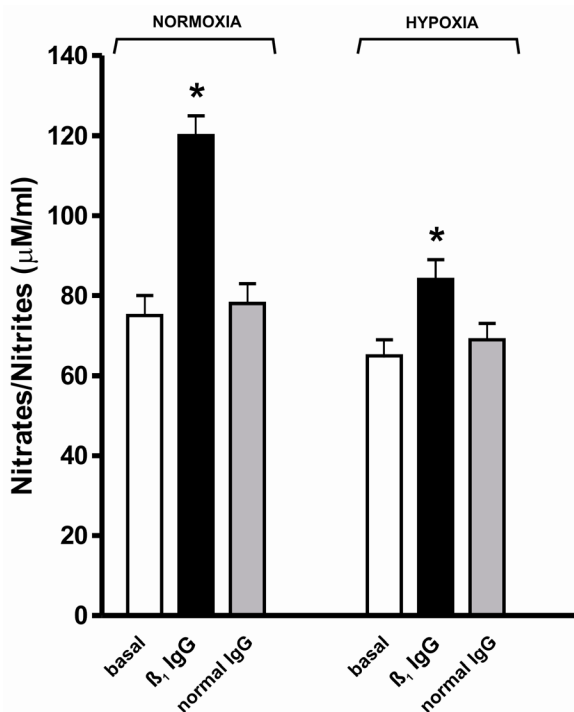
Figure 3. Effects of 1×10^{-8} M β_1 IgG on nitrate/nitrite production. Normal β_1 IgG is shown as control (basal values). Data shown are the mean ± SEM of six experiments in normoxia and hypoxia, performed in duplicate. *P < 0.001 versus basal.
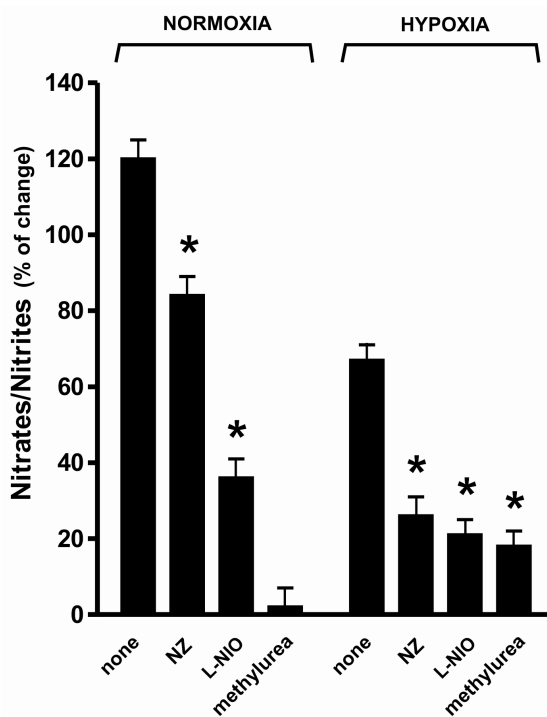

Figure 4. Effect of methylurea 1×10^{-6} M, L-NIO 5×10^{-6} M and NZ 5×10^{-6} M in the presence of 1×10^{-8} M β_1 IgG on nitrate/nitrites values. Data shown are expressed in percent of change values and represent the mean ± SEM of six experiments in normoxia and hypoxia, performed by duplicate. *P < 0.001 versus basal values.

In order to assess the action of action of β_1 IgG on enzymatic pathways coupled to β_1 rat atria adrenoreceptors were studied *i.e.* biological effect (contractility) expressed as dF/dt (g/s), cGMP (pmol/g tissue wet wt) production and PKC (pmol/min/mg protein) activity in atria exposed to β_1 IgG. Also, in comparative form we studied the action of β_1 agonist ISO (1×10^{-7} M).

As shown in **Table 1**, β_1 IgG increased dF/dt and the PKC translocation during normoxia and hypoxia, as well as cGMP accumulation in hypoxia conditions while normal β_1 IgG had no effect.

Furthermore, the increment induced by the autoantibody was neutralized after preincubating the IgG with the β_1 synthetic peptide (5×10^{-5} M) and Atenolol (1×10^{-7} M) an authentic β_1 antagonist. In comparison, all these effects of β_1 IgG resembled those of the authentic adrenoceptor agonist ISO (**Table 2**).

4. Discussion

The study of cardiac normoxia and hypoxia conditions on β_1 IgG from serum of patients with periodontitis in relationship with the influence of NO production and NOS expression in rat atria myocardium was analyzed.

Our results show that our model of normoxia/hypoxia significantly raised nNOS, eNOS and iNOS mRNA and protein levels and atria contractility, as well as activation of enzymatic activity (guanylate cyclase and PKC) and

Table 1. Effect of β_1 IgG upon rat atria during normoxia and hypoxia alone or in the present of inhibitors.

Additions and experimental conditions	dF/dt (g/s)	PKC (pmol/min/mg/protein)	cGMP (pmol/g tissue wet wt)
Basal normoxia	7.6 ± 0.6	1.4 ± 0.1	42 ± 3.2
β_1 IgG normoxia	11.4 ± 0.9*	6.2 ± 0.3*	45 ± 5.1
β_1 IgG + atenolol normoxia	7.4 ± 0.7	1.7 ± 0.2	47 ± 4.2
β_1 IgG + $\beta_1$syntetic peptide normoxia	7.8 ± 0.7	1.6 ± 0.6	46 ± 4.2
Normal β_1 IgG normoxia	7.5 ± 0.4	1.6 ± 0.2	48 ± 4.2
Basal hypoxia	4.6 ± 0.2	1.2 ± 0.1	43 ± 2.8
β_1 IgG hypoxia	6.8 ± 0.4*	4..6 ± 0.2*	44 ± 3.7
β_1 IgG + atenolol hypoxia	4.4 ± 0.3	1.4 ± 0.3	45 ± 2.6
β_1 IgG + β_1 syntetic peptide hypoxia	4.1 ± 0.4	1.6 ± 0.5	48 ± 5.2
Normal β_1 IgG hypoxia	3.9 ± 0.5	1.7 ± 0.4	47 ± 6.2

Values are mean ± SEM of five experiments in each group performed in duplicate. Atria contractility was expressed as dF/dt. Enzyme activities were measured after incubation during 2 hours rat atria in presence of β_1 IgG (1×10^{-8} M) or Normal β_1 IgG (1×10^{-7} M) with or without inhibitors: atenolol 1×10^{-7} M (β_1 adrenergic antagonist) and β_1 syntetic peptide 5×10^{-5} M in normoxia and hypoxia experimental conditions. *P < 0.01 comparing with basal normoxia and basal hypoxia.

Table 2. Effect of ISO upon rat atria during normoxia and hypoxia alone or in the present of inhibitors.

Additions and experimental conditions	dF/dt (g/s)	PKC (pmol/min/mg/protein)	NOS (pmol/g tissue wet wt)	Nitritos (μM)	cGMP (pmol/g tissue wet wt)
Basal normoxia	8.6 ± 0.6	1.3 ± 0.2	50 ± 4	75 ± 7	42 ± 3.2
ISO normoxia	13.4 ± 0.9*	7.2 ± 0.4*	200 ± 18*	140 ± 12*	99 ± 11.2*
ISO + atenolol normoxia	8.4 ± 0.7	1.7 ± 0.5	65 ± 6	77 ± 8	47 ± 5.1
Basal hypoxia	5.6 ± 0.2	1.4 ± 0.2	52 ± 5	67 ± 6	43 ± 2.8
ISO hypoxia	7.1 ± 0.5*	4.8 ± 0.3*	220 ± 15*	90 ± 8*	104 ± 10.1*
ISO + atenolol hypoxia	5.4 ± 0.4	1.3 ± 0.3	109 ± 9	70 ± 5	49 ± 5.9

Values are mean ± SEM of five experiments in each group performed in duplicate. Atria contractility was expressed as dF/dt. Enzyme activities were measured after incubation during 2 hours rat atria in presence of ISO (1×10^{-7} M) with or without atenolol 1×10^{-7} M (β_1 adrenergic antagonist) in normoxia and hypoxia experimental conditions. *P < 0.01 comparing with basal normoxia and basal hypoxia.

the levels of total nitrate/nitrites in hypoxia, implying a crucial moment in the changes that the NO/NOS system undergoes during myocardial hypoxia.

The molecular interactions of β_1 IgG from sera of periodontitis patients with myocardial β_1 adrenergic receptors have the capacity to generate the proinflammatory substance NO, that could act as inducer of iNOS mRNA during hypoxia.

The major finding of this work was to demonstration that β_1 IgG antibodies behaving as adrenergic agonist have the capacity to alter the rate of transcription of specific proinflammatory target genes, triggering the increase amounts of NO during hypoxia conditions by iNOS isoforms in response to receptor-mediated signalling events at the cell membrane. The transcription is rapidly induced following receptor activation and therefore, the target genes can be classified as early gene [33]; since iNOS mRNA and the others NOS isoforms in early stages of hypoxia conditions, appears after the activation of β_1 cardiac adrenergic receptors by the autoantibodies (β_1 IgG) during two hours. The iNOS early gene in hypoxia could play an important role in coupling receptor stimulation to long term responses [34] and the autoantibody might play a role in the pathophysiological mechanisms underlying the relevant inflammatory process described in periodontal disease as well as, a same protective protection to the myocardium during early hypoxia conditions.

However, the large amounts of proinflammatory and cytotoxic NO by the activation of iNOS in hypoxia, provoking and resulting in inflammation, immunological alterations and cytotoxic myocardial tissue damage [12]. The proinflammatory mediator (NO) and its products (nitrites) are induced in ischemic heart and in heart failure contributing to inflammatory cell infiltration [35]. We reported previously [36] that the inflammatory process described in atria myocardium in the presence of β_1 IgG from periodontitis patients might be attributed in part, to autoantibodies fixation on sarcolemma, that by interacting with atria β_1 adrenergic receptors, triggers not only the release of NO but also generate PGE2 production via COX-2 activation and expression [36]. Furthermore, there are evidence that the activation of COX-2 was preceded by NOS activation and this study rise the possibility that COX-2 enzyme represents an important endogenous "receptor" target for different NOS isoforms [32].

The nNOS and iNOS is activated by β_1 IgG during hypoxia with major participation of iNOS and this iNOS signalling system appears to be a key factor for periodontitis IgG-induced iNOS products such as NO, nitrites/nitrates and cGMP accompanied by an increase PKC translocation to the cardiac membrane. Moreover, PKC translocation had been previously proposed as a transduction mechanism of ischemic preconditioning mediated the activation of phosphatidylinositol-3-kinase, upstream PKC and NO have been reported [37] [38]. Also, cGMP have been identified as potential mediators of hear precondition [39]. It is known that COX-2 is to be a co-inducer together with iNOS in cardiac myocytes in response to stress [40].

Regarding iNOS, while some authors have reported a greater expression in the hypoxic rat heart [7], others have shown that iNOS is substantially down regulated by hypoxia in the ventricles [41]. Moreover, it has been reported that hypoxia alone has no effect on iNOS gene expression in cardiomyocytes [42]. On the other hand, our results are in accordance with previous report showing that nNOS and eNOS expression is much less prominent in rat atria [8] and hypoxia has no significant impact on cardiac nNOS expression [42].

Thus, all the changes that the NO/NOS undergoes during hypoxia by the action of the autoantibody (β_1 IgG) could be involved in the adaptation of the heart to such situation. It is noteworthy that all the parameters analysed in the present work (nitrites/nitrates, cGMP, PKC) by the action of the β_1 IgG, increased at the onset of short time rat atria hypoxia, implying a crucial moment in the heart adaptation phenomena to be maintain heart contractility and its behaviour.

5. Conclusion

It should be noted that the association of NOS isoforms in normoxia and its changes in hypoxia by the action of β_1 IgG, the NO production, the cGMP increasing accumulation, the increasing concentration of nitrates/nitrites and PKC translocation, is a machine that might be useful and helps to maintain heart function and to prevent necrosis and subsequent loss of heart function.

Acknowledgements

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