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Pro-apoptotic effect of anti-β_1-adrenergic receptor antibodies in periodontitis patients

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ABSTRACT

An anti-β_1-adrenergic antibody from the sera of periodontitis patients (anti-β_1-AR IgG) against the second extracellular loop of the human β_1-adrenoceptor (β_1-AR) has been shown to cause rat atria apoptosis. The anti-β_1-AR IgG binds and activates atria β_1-AR, increasing the intracellular calcium concentration, which, in turn, activates caspases-3, -8, and -9. The β_1-AR and the post-receptor activation of calcium/calmodulin (CaM) lead to increased inducible nitric oxide synthase (iNOS) activity, with an increase in cyclic GMP (cGMP) accumulation as well as increased JNK phosphorylation and cyclic AMP (cAMP) production. We also observed an apoptotic effect of anti-β_1-AR IgG, with increased generation of PGE_2. Comparatively, xamoterol, an authentic β_1-AR agonist, mimicked the autoantibody effect on rat atria β_1-AR apoptosis. Our results suggest that autoantibodies from the sera of periodontitis patients bind and interact with rat atria β_1-AR, provoking apoptosis. This implicates a series of modulatory cardiac signaling events that could alter normal heart function and may occur with chronic stimulation of the atria β_1-AR, which could lead to heart failure. These results suggest an important link between periodontitis and cardiovascular disease.

1. Introduction

Autoimmune cardiomyopathy has gained increased recognition as an important cause of human dilated cardiomyopathy. Among the anti-cardiac autoantibodies, antibodies against the β_1-adrenoceptor (β_1-AR) have been detected in patients with idiopathic dilated cardiomyopathy [1,2], chagasic myocardiopathy [3,4] and in patients suffering from chronic periodontal disease, with or without myocardial failure [5,6].

Anti-β_1-adrenoceptor IgG (anti-β_1-AR IgG) appears to be pathophysiologically important because these antibodies not only exert direct apoptotic and negative inotropic effects in cultured cardiomyocytes [7–9] and a positive inotropic effect in isolated mice and rat atria [10,11], but also induce dilated cardiomyopathy in animals following transfer of sera [7] or IgG [12] from diseased animals. Because the effects of anti-β_1-AR IgG appear to be mediated via the myocardium β_1-adrenoceptors (β_1-AR) and these antibodies are capable of binding and interacting with myocardial β_1-AR, there is also a functional link to β_1-AR-mediated activation of cardiac adenylate cyclase [13] and calcium/calmodulin (CaM) complex [14,15] and other signaling mechanisms identified as important second messengers regulating cardiac function [16].

The positive inotropic effect of β_1-AR stimulation is an effective measure for maintaining cardiac output by activation of protein kinase A through G-proteins, adenylate cyclase, and cyclic AMP [17]. However, long-term stimulation of β_1-AR can lead to deterioration in cardiac function and one mechanism that may contribute to this is thought to be the induction of apoptosis on β_1-AR stimulation [18]. Norepinephrine and isoproterenol stimulate β_1-AR and induce apoptosis in rat cardiomyocytes [19] and the role of nitric oxide (NO) as a messenger, and also as an effector molecule, affects programmed cell death through cyclic guanosine monophosphate (cGMP)-dependent and -independent pathways [20].

Apoptosis is a highly regulated process in which pro-death and pro-survival cell signals are regulated and integrated to determine the fate of a cell. In fact, in the mitochondrial-mediated intrinsic pathway, stimuli lead to the release of mitochondrial membrane proteins that assemble into an apoptosome, which ultimately activates caspase that are mediators of cell death [21,22]. Potent proteases, like caspase-3, break up sub-cellular cytoplasmic and nuclear proteins. Caspase-8, associated with cytokines, is associated with the extracellular pathway, which follows mitochondrial events via the release of cytochrome *c* into the cytoplasm and activation of caspase-9. All these pathways finally converge on the terminal or executioner caspase, which mediate the final steps of apoptosis [23].

Cardiac apoptosis plays a key role in the pathogenesis of a variety of cardiovascular diseases, such as myocardial infarction [24], dilated

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cardiomyopathy [25], autoimmune cardiomyopathy [26] and heart failure [27]. It is also a highly regulated program of cell death and inhibition of this process is cardio protective under many conditions.

Periodontitis is characterized by gingival inflammation and periodontopathic bacteria generate immunological inflammatory responses. Periodontitis is a key risk factor for the onset of cardiovascular disease [24–26]. Recently, we reported that in the sera of periodontitis patients we found autoantibodies against atria cardiac β_1-AR that were able to mimic the effect of an authentic β_1-AR agonist acting on atria β_1-AR [5,6]. However, the release of host-derived inflammatory mediators, such as cytokines from chronically inflamed periodontal tissues, into the circulation together with the sera anti-β_1-AR IgG, may provide a link between periodontal disease and cardiovascular disease [27,28].

In this paper, we will discuss (a) the effect of anti-β_1-AR IgG acting on β_1-AR in rat atria and its capacity to activate caspase pathway, (b) molecular signals involved in anti-β_1-AR IgG – β_1-AR-stimulated myocardium apoptosis and increased cAMP production and JNK phosphorylation, and (c) the role of anti-β_1-AR IgG in the release of inflammatory mediators (PGE_2, NO, cGMP) that participate in atria β_1-AR-stimulated cardiomyocytes apoptosis.

2. Experimental procedure

2.1. Ethical approval of the study protocol

The study protocol complied with the tenets of the Declaration of Helsinki and the rules established by the Ethics Committee of the University of Buenos Aires (Buenos Aires, Argentina). All subjects provided written informed consent.

2.2. Patients

The study group consisted of 16 adult patients with periodontitis who were attending the Periodontology Clinic from the metropolitan area of Buenos Aires. The mean age was 41 (range, 32-50) years. Healthy subjects were used as controls (15 male subjects) with a mean age of 38 (range, 30-46) years. The assessment of clinical parameters was carried out by a trained periodontist following the criteria based on clinical parameters and the severity of periodontal tissue destruction [29]. The characteristic clinical signs of periodontitis included loss of clinical attachment, horizontal and/or 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 in 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. No subject (periodontal patient or healthy individual) had any systemic illness and they were all never-smokers. Patients with periodontitis had not received periodontal treatment or antibiotics within the preceding 5 months or any anti-inflammatory drug within 3 weeks prior to the study. The clinical characteristics of the study population and the healthy subjects (controls) are shown in Table 1. Additionally, pocket probing depth (PPD) and clinical attachment loss (CAL) are shown in Table 2.

Table 1
Characteristics of the study populations.

Demography and risk factors	Periodontitis patients ($n=16$)	Healthy subjects ($n=15$)
Gender		
Male	14	14
Female	2	1
Education level		
Elementary school	14	13
High school	2	2
BMI (range kg/m^2)	From 20 to 26	From 19 to 24
Measure blood pressure (mm Hg)		
Measure SBP (mean ± SD)	133 ± 20	120 ± 14
Measure DBP (mean ± SD)	85 ± 12	78 ± 10
Laboratory examination		
Cholesterol total (mean ± SD), mg/dL	162 ± 19	178 ± 22
LDL (mean ± SD), mg/dL	119 ± 12	128 ± 21
HDL (mean ± SD), mg/dL	32 ± 11	30 ± 12

BMI: body mass index (range ≥ 27 kg/m^2); SBP: systolic blood pressure. DBP: diastolic blood pressure; LDL: low-density lipoprotein; HDL: high-density lipoprotein.

2.3. Human sera and IgG purification

Sera and the corresponding IgG were obtained from patients with periodontitis and normal individuals. Blood (6 mL) was obtained by venipuncture and allowed to clot at room temperature. Serum was then separated by centrifugation (2000 *g*) and stored at −80 °C until used in assays. IgG was obtained by precipitation with ammonium sulfate at 50%, followed by three washes and re-precipitation with 33% ammonium sulfate. The resulting precipitate was subjected to chromatography on DEAE-cellulose, equilibrated with 10 mM phosphate buffer (pH 8). The eluted peaks were concentrated by ultrafiltration to 10 mg protein/mL. Control immune electrophoresis with goat anti-human total serum and goat non-specific anti-human IgG showed only one precipitin line.

2.4. F(ab′)$_2$ isolation

The isolation of F(ab′)$_2$ from normal and periodontal IgG was performed as described previously [30]. Briefly, normal and periodontal IgG were subjected to pepsin digestion, incubated overnight at 37 °C, pH 4.5, and centrifuged. The supernatants were adjusted to pH 7.4 and dialyzed overnight with phosphate-buffered saline (PBS). The dialysate was then applied to Sephadex G-100 columns equilibrated with PBS. Two significant peaks were obtained and the activities were examined. The second peak contained the F(ab′)$_2$ activity, assayed by immune electrophoresis using rabbit anti-mouse immunoglobulin sera.

2.5. β_1-adrenergic synthetic peptide

A 27-mer peptide, H-W-W-R-A-E-S-D-E-A-R-R-C-Y-N-D-P-K-C-C-D-F-V-T-N-R-C, corresponding to the amino acid sequence of the second extracellular loop of human β_1-AR was synthesized using Fmoc amino acids activated with a 1-hydroxybenzotriazole/dicyclohexylcarbodiimide (HOBt/DCC) strategy using an automatic peptide synthesizer (Model 431A; Applied Biosystems, Menlo Park, CA, USA). The peptide was desalted and purified by high-performance liquid chromatography (HPLC). It was then subjected to amino-terminal sequence analysis by automated Edman degradation (470A Sequencer; Applied Biosystems). An unrelated 27-mer peptide, S-G-S-G-S-G-S-G-S-G-S-G-S-G-S-G-S-G-S-G-S-G-S-G-S-G-S-G-S, was also synthesized as a negative control.

Table 2
Periodontitis selection index.

Parameters	Numerical ranges
PPD	≥6 mm
CAL	≥6 mm

PPD: pocket probing depth, CAL: clinical attachment loss.

2.6. Determination of apoptosis

2.6.1. TUNEL assay

Rat atria (10 mg) were incubated in 1 mL Krebs-Ringer bicarbonate (KRB) for 60 min in oxygen at 37 °C, pH 7.4, and the anti-β_1-AR IgG or the β_1-AR agonist xamoterol was added in the last 40 min. When a blocker was used, it was added 20 min before the addition of the antibody or xamoterol. All tissue specimens were fixed in 10% formaldehyde solution for 24 h. After fixation they were processed routinely and embedded in paraffin. Sections (5 μm) were taken onto polylysine-coated slides. Then, the slides were deparaffinized in the usual manner (oven at 65 °C for 1 h, graded alcohols, and finally xylene treatment). In the present study, a terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick and labeling (TUNEL) assay was used to identify cells containing fragmented nuclei, an indicator of apoptosis. After deparaffinization, a TUNEL (Roche Molecular Biochemicals, Mannheim, Germany) kit was used according to the manufacturer's protocol [31]. Briefly, the following sequence was used for the TUNEL method: inhibition of endogenous peroxidase in 0.3% H_2O_2 in methanol for 30 min, rinse in tap water and soak in 50 mM Tris-buffered saline, pH 7.6, pretreatment in a microwave for 15 min in sodium citrate buffer, rinse in tap water and soak in TdT buffer for 5 min, incubate with TdT mixture at 37 °C for 60 min, rinse in 10 mM phosphate-buffered saline (PBS), pH 7.2, incubate with peroxidase-labeled streptavidin for 15 min, rinse in 10 mM PBS, react in the diaminobenzidine solution for 10 min, rinse in tap water, stain with Mayer's hematoxylin for 1 min, rinse in

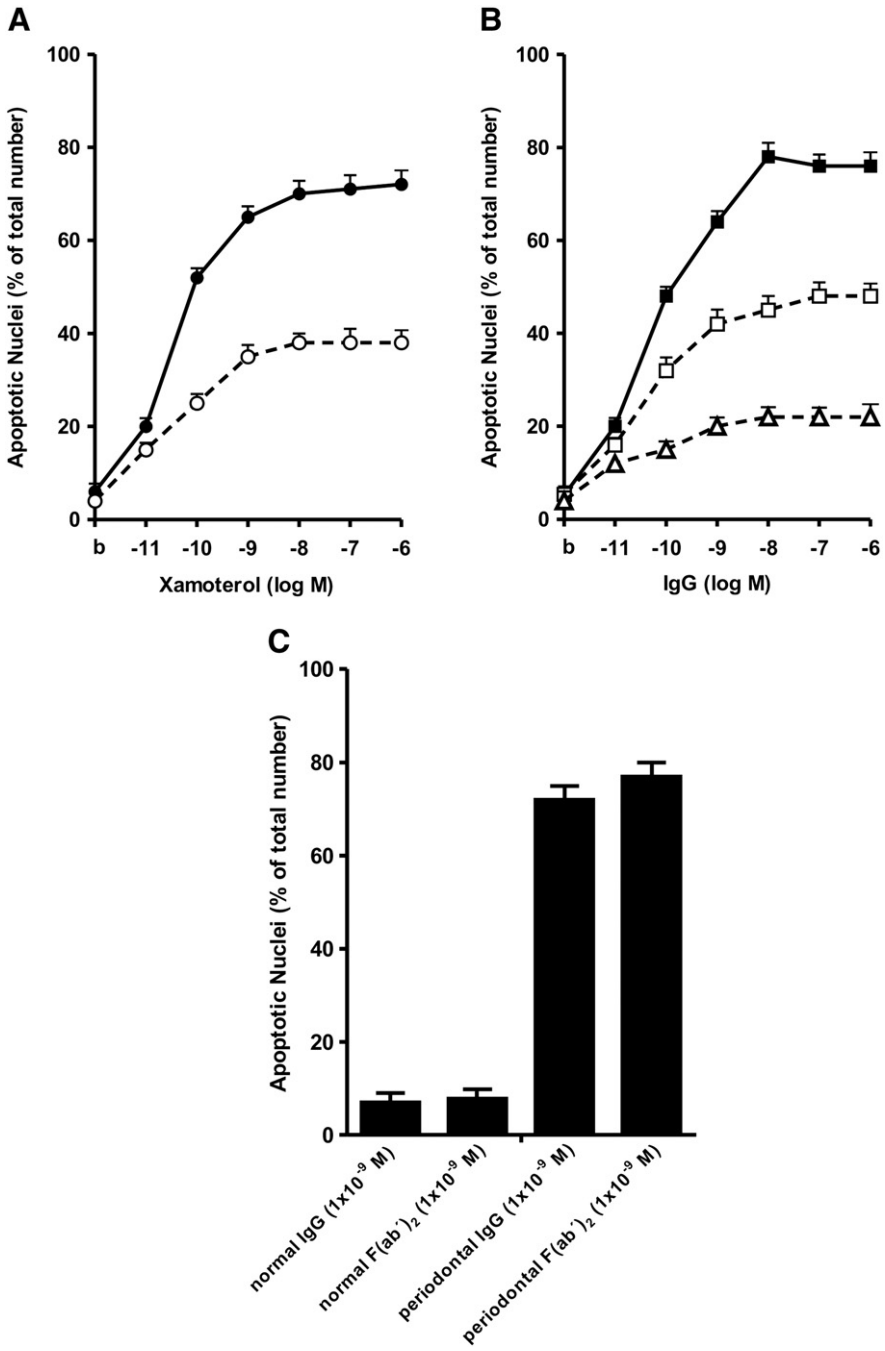

Fig. 1. The effect of xamoterol (A) and periodontal IgG (B) on rat atria apoptosis, was assessed by the TUNEL assay, as described in the Experimental procedure and (C) the effect of periodontal and normal F(ab′)$_2$ on rat atria apoptosis, as assessed by the TUNEL assay. Concentration–response curve of xamoterol (•—•), atenolol 1×10^{-7} M + xamoterol (○---○), periodontal IgG (■—■), atenolol 1×10^{-7} M + periodontal IgG (□---□) and normal IgG (healthy subjects as control) (△---△). Values are means ± SEM of $n=6$ in each group, performed in duplicate.

tap water, dehydrate to xylene and mount. Tissues were visualized using an Axiolab Carl Zeiss microscope. All photographs were taken at 100× magnification using a Canon PowerShot G5 camera. Results were expressed as the percentage (%) of TUNEL positive cells of at least 15 fields examined.

2.6.2. Caspase assay

Caspase-3, -8, and -9 activities were measured using the CaspACE Assay System Colorimetric Kit (Promega Corp., Madison, WI). Rat atria (10 mg) were incubated in 1 mL Krebs-Ringer bicarbonate (KRB) for 60 min in oxygen at 37 °C, pH 7.4, and the anti-β_1-AR IgG or the β_1-AR agonist xamoterol was added in the last 40 min. When a blocker was used, it was added 20 min before the addition of the antibody or xamoterol. After incubation, the atria tissue was homogenized in 1 mL of lysis buffer and centrifuged (1500 *g*, 5 min, 4 °C) and the supernatant was centrifuged at 2000 rpm. The supernatant was used to determine the caspase-3, -8, and -9 activities, measured by the cleavage of the colorimetric substrate Ac-DFVD-AMC, according to the manufacturer's instructions. When caspase-specific inhibitor agents and enzymatic inhibitors were used, they were added 20 min before the addition of the antibody or xamoterol.

2.6.3. JNK assay

JNK activity was assessed using a recombinant protein fragment of c-Jun as an affinity ligand and the substrate glutathione S transferase (GST-c-Jun[1–79]) [32]. Rat atria tissue was prepared as described above, and the supernatant obtained was incubated for 60 min at 48 °C with 20 μL of a 25% (v/v) slurry of glutathione Sepharose (Pharmacia-LKB), pre-coupled to GST-c-Jun (5 mg of protein). Beads were collected by centrifugation and washed twice in 200 μL of lysis buffer and twice in 200 μL of kinase buffer. Reactions were initiated by addition of 40 μL of kinase buffer containing 20 mM [γ-^{32}P]ATP (2.5 mCi/nmol). After incubation for 20 min at 30 °C, reactions were terminated by addition of 40 μL of Laemmli sample buffer and boiling. Phosphorylated proteins were resolved by PAGE through 12% acrylamide in the presence of 0.1% SDS, stained with Coomassie Blue R250, and visualized by autoradiography. Radioactivity incorporated into GST-c-Jun was quantified by liquid scintillation counting of the excised bands.

2.7. Cyclic nucleotides assay (cAMP-cGMP)

Rat atria (10 mg) were incubated in 1 mL KRB for 30 min, and the anti-β_1-AR IgG and xamoterol were added in the last 15 min. When a blocker was used, it was added 25 min before the addition of the antibody or the β_1 adrenergic agonist. After incubation, atria tissue was homogenized in 2 mL of absolute ethanol and centrifuged (6000 *g*, 15 min, 4 °C). Pellets were then re-homogenized in ethanol–water (2:1) and re-centrifuged. The supernatant was collected and evaporated to dryness. Cyclic AMP and cyclic GMP in the residue were

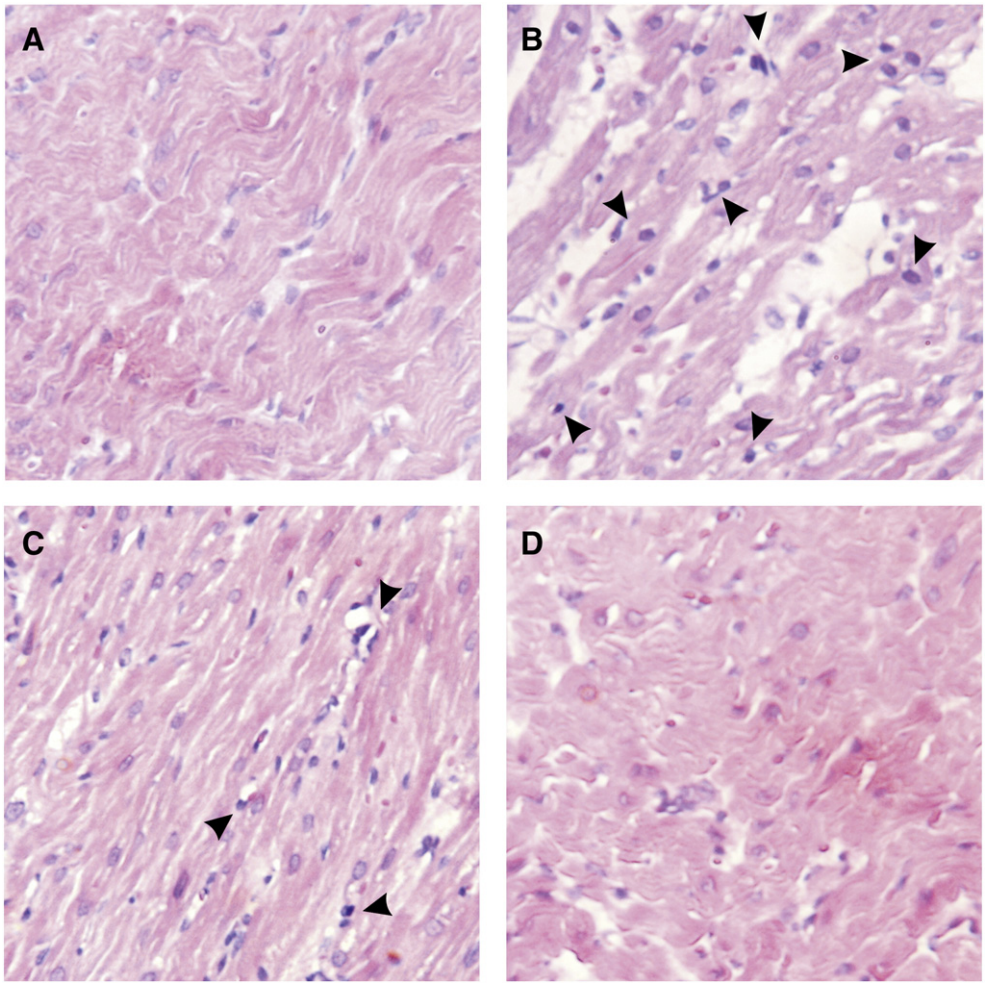

Fig. 2. Xamoterol (1×10^{-8} M), a β_1-AR specific agonist-induced apoptosis in isolated rat atria. Apoptosis was analyzed by TUNEL assay: A: absence of TUNEL-positive cells; B: increased TUNEL-positive cells (arrows); C: reduction of TUNEL-positive cells when atenolol (1×10^{-6} M) (arrows) was added previously to xamoterol and D: absence of TUNEL-positive cells is observed when atrium was treated only with atenolol. Pictures were taken at 100× magnification.

dissolved in 400 μL of 0.05 M sodium acetate buffer (pH 6.2). For the determination of nucleotides, we used ELISA employing the protocol for the production of cAMP or cGMP from Amersham Biosciences (Piscataway, NJ, USA). Results are expressed as picomoles per milligram of wet weight of tissue (pmol/mg tissue wet weight).

2.8. PGE_2 assay

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. The anti-β_1-AR IgG or the β_1-AR agonist xamoterol was added 40 min before the end of incubation period and blockers 20 min before the addition of the IgG or xamoterol. Tissues were then homogenized in a 1.5-mL polypropylene microcentrifuge tube. The remaining procedures were those indicated in the protocol of the Prostaglandin E_2 Biotrak Enzyme Immuno Assay (ELISA) System (Amersham Biosciences, Piscataway, NJ, USA). The PGE_2 results were expressed as picogram/milligram of tissue wet weight.

2.9. Nitric oxide synthase assay

Nitric oxide synthase (NOS) activity was measured in rat atria by the production of [U–^{14}C] citrulline from [U–^{14}C] arginine, when the tissue was 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 xamoterol or anti-β_1-AR IgG on carbogen at 37 °C. Tissues were then homogenized using an Ultraturrax. After centrifugation (2000 *g*, 10 min, 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 95% by 0.5 mmol/L N^G-monomethyl-L-arginine (L-NMMA).

2.10. Drugs

Stock solutions were prepared fresh in the corresponding buffers. Drugs were diluted in the bath to achieve the final concentrations stated in the text. Xamoterol, atenolol, W-7, verapamil, indomethacin (INDO), ODQ, and N^G-monomethyl-L-arginine (L-NMMA) were obtained from Sigma-Aldrich (St. Louis, MO, USA) and (S)-methylisothiourea sulfate (methylurea), SQ22535, and BI-73D3 were from Tocris Bioscience (Minneapolis, MN, USA).

2.11. Statistical analyses

Student's *t*-test for unpaired values was used to determine the levels of significance. Analysis of variance (ANOVA) and a *post hoc* test (Dunnett's method and Student–Newman–Keuls test) were employed when pair-wise multiple comparison procedures were

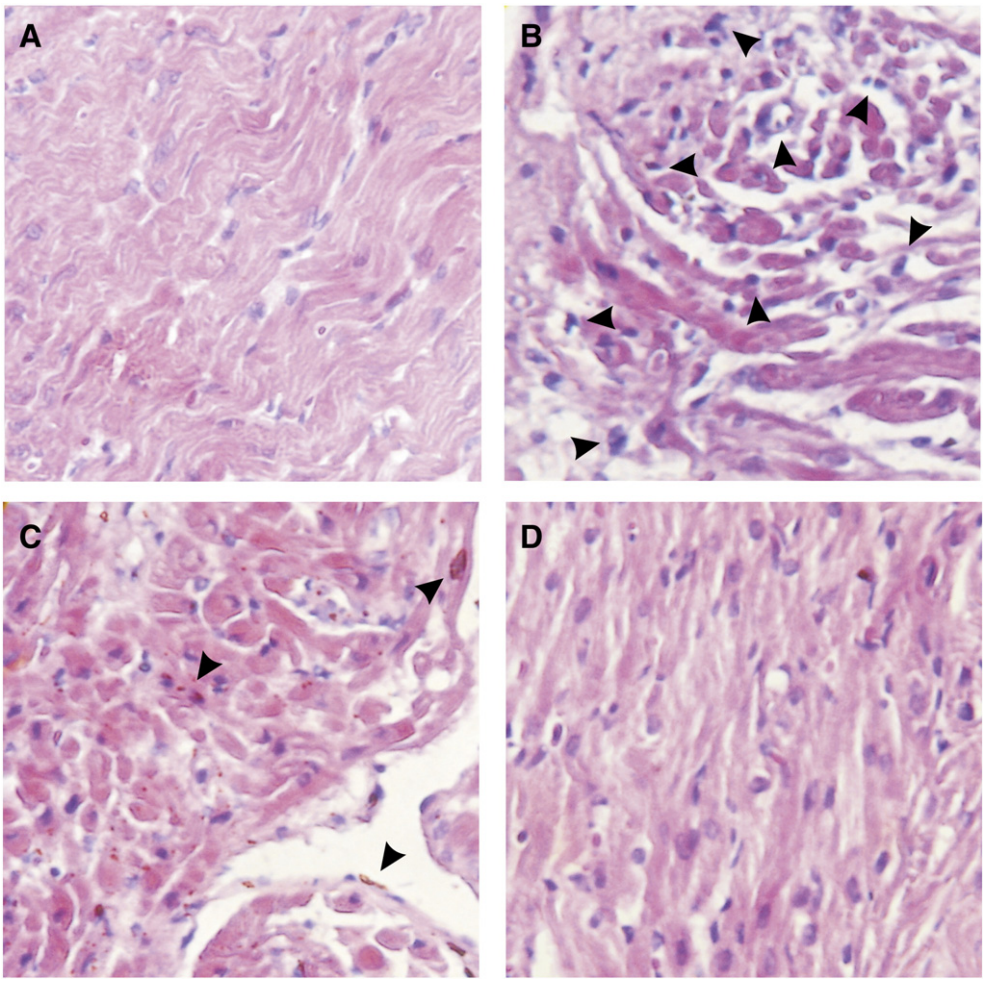

Fig. 3. Periodontal IgG (1×10^{-8} M)-induced apoptosis in isolated rat atria. Apoptosis was analyzed by TUNEL assay: A: showed almost absence of TUNEL-positive cells; B: increased TUNEL-positive cells (arrows); C: reduction of TUNEL-positive cells when a β_1 synthetic peptide (5×10^{-5} M) (arrows) was added previously to periodontal IgG and D: absence of TUNEL-positive cells is observed when atrium was treated only with β_1 synthetic peptide. Pictures were taken at 100× magnification.

necessary. Differences between means were considered significant at $P<0.05$.

3. Results

3.1. Rat atria apoptotic nuclei

We initially determined comparatively the stimulatory effect of the different concentrations of xamoterol (a β_1-specific agonist; Fig. 1A) and periodontal anti-β_1-AR IgG (Fig. 1B) in rat atria apoptotic nuclei. We found that xamoterol caused a marked increase in the number of apoptotic atria nuclei, as shown by the TUNEL assay (Fig. 1A) and maximal effect of the β_1-specific agonist was obtained at 1×10^{-8} M. Fig. 1A also shows the lack of action of xamoterol in the presence of 1×10^{-7} M atenolol, a β_1-specific antagonist. Fig. 1B shows the increase in the number of atria apoptotic nuclei in the presence of increasing concentrations of IgG; IgG reached a maximum capacity for stimulation at 1×10^{-9} M. As in Fig. 1A, atenolol abrogated the stimulatory capacity of periodontal IgG to increase atria apoptotic nuclei. Normal IgG, used as control, was ineffective in the system (Fig. 1B). The effect of periodontal IgG was specific, because it was due to the $F(ab')_2$ fraction of IgG. Normal $F(ab')_2$ was ineffective in the system (Fig. 1C). Figs. 2 and 3 showed when rat atria were examined by TUNEL staining for the presence of apoptotic nuclei in the presence of xamoterol and anti-β_1-AR IgG respectively. It can be seen that xamoterol-treated atria exhibited an increased apoptotic TUNEL-positive nucleus (Fig. 2B) and an important reduction of them was detected when atenolol (1×10^{-6} M) was added before xamoterol (Fig. 2C). Almost, the absence of TUNEL-positive nuclei was seen in atria without any addition (control) (Fig. 2A) or atria treated with atenolol alone (Fig. 2D). On the other hand, Fig. 3B showed that anti-β_1-AR IgG-treated atria exhibited an increased apoptotic TUNEL-positive nuclei and an important reduction of them was detected when β_1 synthetic peptide (1×10^{-5} M) was added before the antibody (Fig. 3C). Almost, the absence of TUNEL-positive nuclei was seen in atria without any addition (control) (Fig. 3A) or atria treated with β_1 synthetic peptide alone (Fig. 3D).

3.2. Caspase activities

Fig. 4 shows the ability of xamoterol (A) and periodontal IgG (B) to trigger the activation of caspase-3 in a concentration-dependent

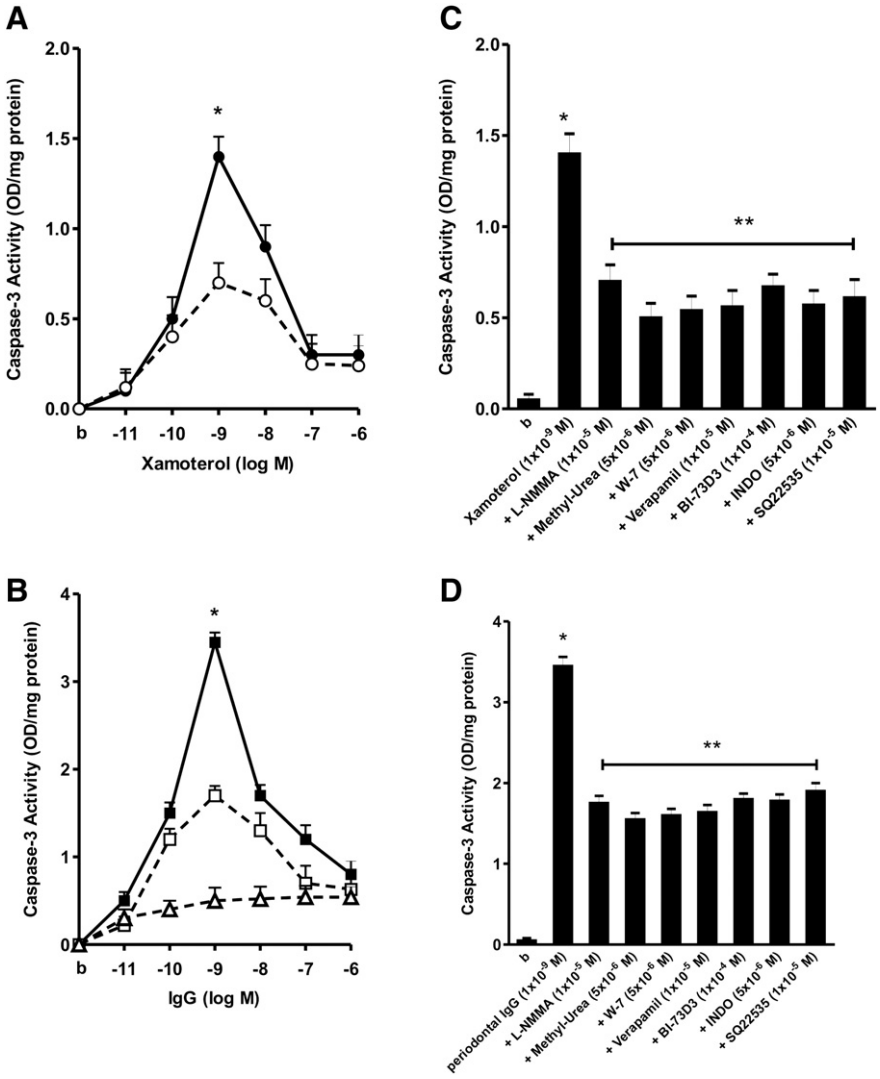

Fig. 4. A: Concentration–response curve of xamoterol alone (•—•) or in the presence of Z-DEVD-FMK 1×10^{-5} M (○---○) and B: periodontal IgG alone (■—■) or in the presence of Z-DEVD-FMK 1×10^{-5} M (□---□) and normal IgG (△---△) inducing an increase in caspase-3 activity. C: Effect of xamoterol alone or in the presence of L-NMMA, methylurea, W-7, verapamil, BI-73D3, INDO and SQ 22535 and D: effect of periodontal IgG alone or in the presence of L-NMMA, methylurea, W-7, verapamil, BI-73D3, INDO and SQ 22535. b: basal values. Values are means ± SEM of $n=8$ in each group, performed in duplicate. *$P<0.001$, versus basal values; **$P<0.0001$, versus xamoterol or periodontal IgG.

manner with 1×10^{-9} M providing a maximal response and decreasing thereafter, raising values significantly higher than basal ones. The presence of Z-DEVD-FMK (1×10^{-5} M), specifically blocked the stimulatory action of xamoterol and periodontal IgG on caspase-3 activity. Normal IgG, as a control, had no effect (Fig. 4A, B). The histogram of Fig. 4 shows a significant reduction in xamoterol-periodontal IgG-induced caspase-3 activation in the presence of L-NMMA (NOS inhibitor), methylurea (iNOS inhibitor), W-7 (CaM inhibitor), verapamil (calcium flux inhibitor), BI-73D3 (JNK inhibitor), INDO (COX inhibitor), and SQ22535 (adenylate cyclase inhibitor). Significant stimulation with xamoterol alone (A) and periodontal IgG alone (B) was also observed.

Fig. 5 shows the ability of xamoterol (A) and periodontal IgG (B) to increase caspase-9 activity in a concentration-dependent manner, with 1×10^{-9} M providing the maximal response and decreasing thereafter. Inhibition of exogenous xamoterol (A) and periodontal IgG (B) inducing caspase-9 activity was observed with Z-LEDH-FMK (1×10^{-5} M). Normal IgG had no effect (Fig. 5B). To determine whether the activation of enzymatic pathways is commonly associated with NOS activation, calcium mobilization, JNK phosphorylation, prostaglandin generation, and adenylate cyclase activation, the actions of different inhibitors that interfere with these enzymes are shown in Fig. 5A (xamoterol) and in Fig. 5B (periodontal IgG). As can be seen, all the enzymatic inhibitors blunted the stimulatory effect of both xamoterol and anti-β_1-AR IgG, pointing to participation of NO, calcium, JNK phosphorylation, PGE_2, and cAMP in these phenomena.

Fig. 6 shows the concentration-dependent increase in caspase-8 activity of xamoterol (A) and periodontal IgG (B). This reaction was blocked by Z-IETD-FMK (1×10^{-5} M). Normal IgG was ineffective in the system (Fig. 6B). In the histogram, L-NMMA, methylurea, W-7, verapamil, and BI-73D3 significantly blunted the actions of xamoterol (Fig. 6A) and periodontal IgG (Fig. 6B) on caspase-8 activity, indicating the involvement of NO, CaM complex, and JNK phosphorylation. The addition of INDO or SQ 22535 before xamoterol or periodontal IgG was added had no influence on casapase-8 activity.

Fig. 7 shows the increased immunoreactivity with 1×10^{-8} M xamoterol (A) and 1×10^{-9} M (B) on caspase activity. Furthermore, a β_1 synthetic peptide (5×10^{-5} M) inhibited periodontal IgG immunoreactivity when periodontal IgG was absorbed with the β_1 synthetic peptide for 30 min at 37 °C and then added into the microtiter

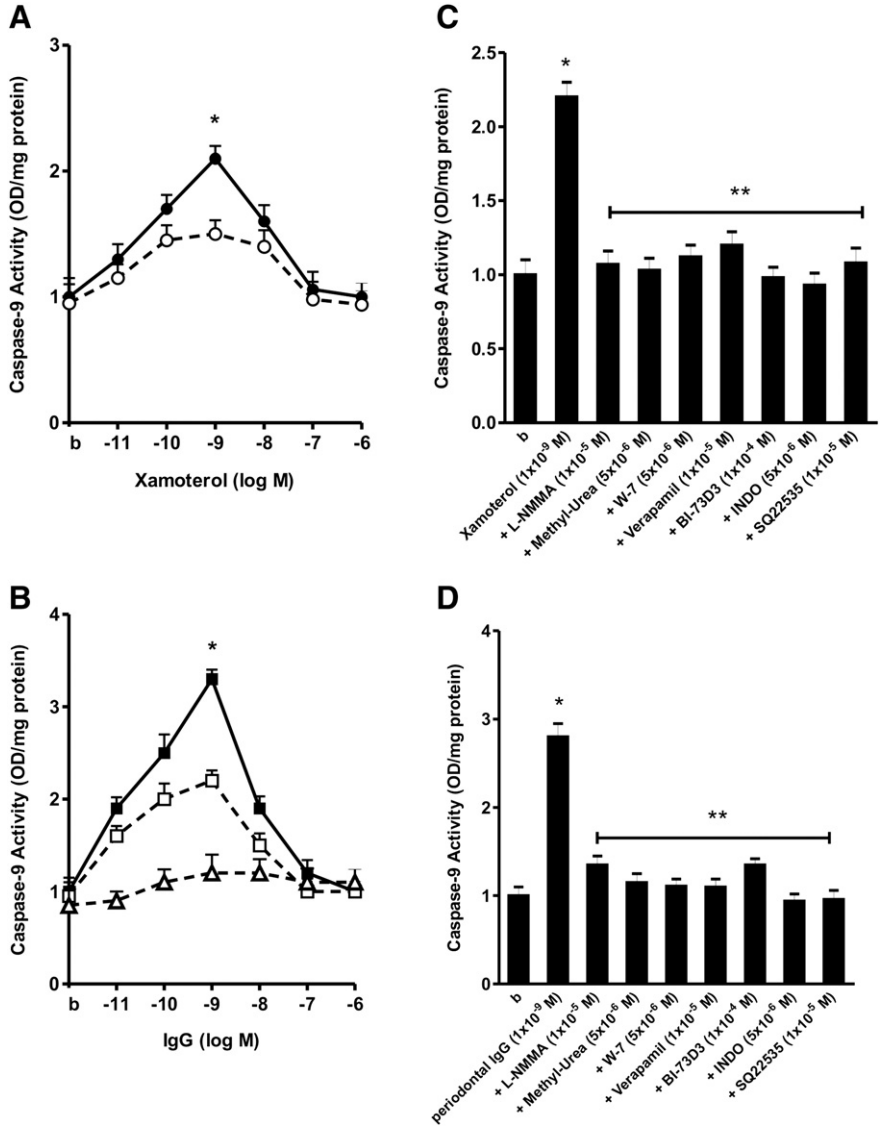

Fig. 5. A: Concentration–response curve of xamoterol alone (•—•) or in the presence of Z-LEHD-FMK 1×10^{-5} M (○---○) and B: periodontal IgG alone (■—■) or in the presence of Z-LEHD-FMK 1×10^{-5} M (□---□) and normal IgG (△---△) inducing an increase in caspase-9 activity. C: Effect of xamoterol alone or in the presence of L-NMMA, methylurea, W-7, verapamil, BI-73D3, INDO and SQ 22535 and D: effect of periodontal IgG alone or in the presence of L-NMMA, methylurea, W-7, verapamil, BI-73D3, INDO and SQ 22535. b: basal values. Values are means ± SEM of $n=8$ in each group, performed in duplicate. *P<0.001, versus basal values; **P<0.0001, versus xamoterol or periodontal IgG.

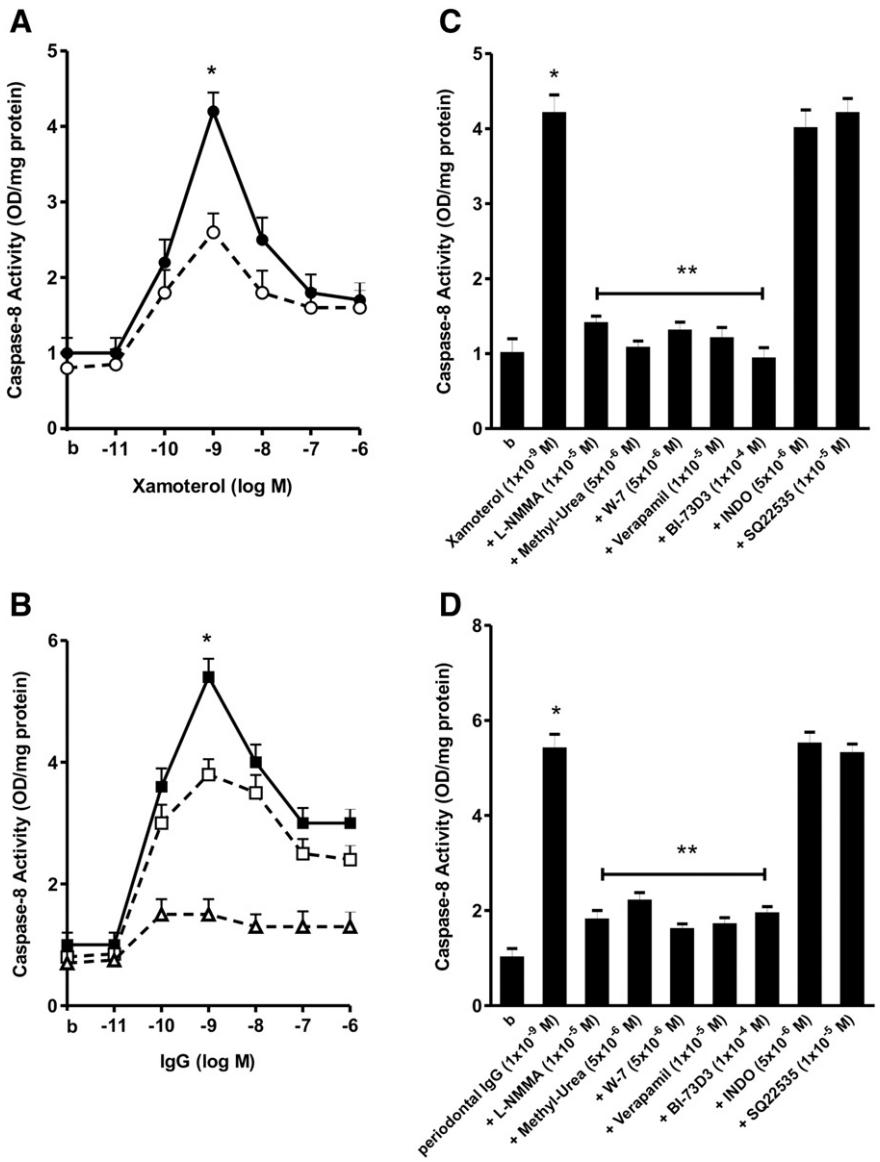

Fig. 6. A: Concentration–response curve of xamoterol alone (•—•) or in the presence of Z-IETD-FMK 1×10^{-5} M (○—○) and B: periodontal IgG alone (■—■) or in the presence of Z-IETD-FMK 1×10^{-5} M (□---□) and normal IgG (△---△) inducing an increase in caspase-8 activity. C: Effect of xamoterol alone or in the presence of L-NMMA, methylurea, W-7, verapamil, BI-73D3, INDO and SQ 22535 and D: effect of periodontal IgG alone or in the presence of L-NMMA, methylurea, W-7, verapamil, BI-73D3, INDO and SQ 22535. b: basal values. Values are means ± SEM of $n=8$ in each group, performed in duplicate. *P<0.001, versus basal values; **P<0.0001, versus xamoterol or periodontal IgG.

plates (Fig. 7B, panel), but the synthetic peptide had no influence on xamoterol (Fig. 7A). On the other hand, atenolol (1×10^{-7} M) prevented the action of both xamoterol (Fig. 7A) and periodontal IgG (Fig. 7B) on caspase activity, confirming the specificity of the β_1-AR reactivity of these autoantibodies. To assess the idea that JNK activation is involved in xamoterol and periodontal IgG-induced apoptosis by β_1-AR-mediated signaling, JNK phosphorylation was assessed in the presence of increasing concentrations of xamoterol, periodontal IgG, and normal IgG. Fig. 8 shows that both xamoterol and periodontal IgG, but not normal IgG, induced activation of JNK enzyme, 1×10^{-7} M providing the maximal effect of xamoterol or periodontal IgG on JNK activation.

It is important to note that the caspase 3, 9 and 8 inhibitors (Z-DEVD-FMK 1×10^{-5} M; Z-LEHD-FMK 1×10^{-5} M, Z-IETD-FMK 1×10^{-5} M) respectively, are able to diminish xamoterol and β1-AR IgG induces apoptosis in rat atria (Table 3). Similar inhibitions upon positive TUNEL nuclei were observed when rat atria were pretreated with 1×10^{-5} M L-NMMA, 5×10^{-5} M methyl-Urea, 5×10^{-5} M W-7, 1×10^{-5} M verapamil, 1×10^{-4} M BI-73D3, 5×10^{-6} M INDO and 1×10^{-5} M SQ22535 (Table 3).

The level of total JNK protein was not modified by xamoterol or periodontal IgG concentration (histogram, Fig. 8). Fig. 9 shows that the maximal capacity to stimulate the activity of the JNK enzyme in the presence of xamoterol or periodontal IgG (both at 1×10^{-7} M) was impaired by 1×10^{-7} M atenolol (β_1-specific antagonist). However, the β_1 synthetic peptide (5×10^{-5} M) blunted only periodontal IgG-stimulated JNK phosphorylation, with no change in xamoterol's effect (Fig. 9).

3.3. Nitric oxide synthase activity (NOS), PGE_2 and cGMP

To examine the atria apoptotic effects of NO, soluble guanylate cyclase and adenylate cyclase activities, and PGE_2, we measured iNOS activity, cAMP and cGMP production, and PGE_2 generation. As shown in Table 4, 1×10^{-8} M xamoterol or 1×10^{-9} M periodontal IgG increased NOS activity, cAMP, cGMP, and PGE_2 production. This

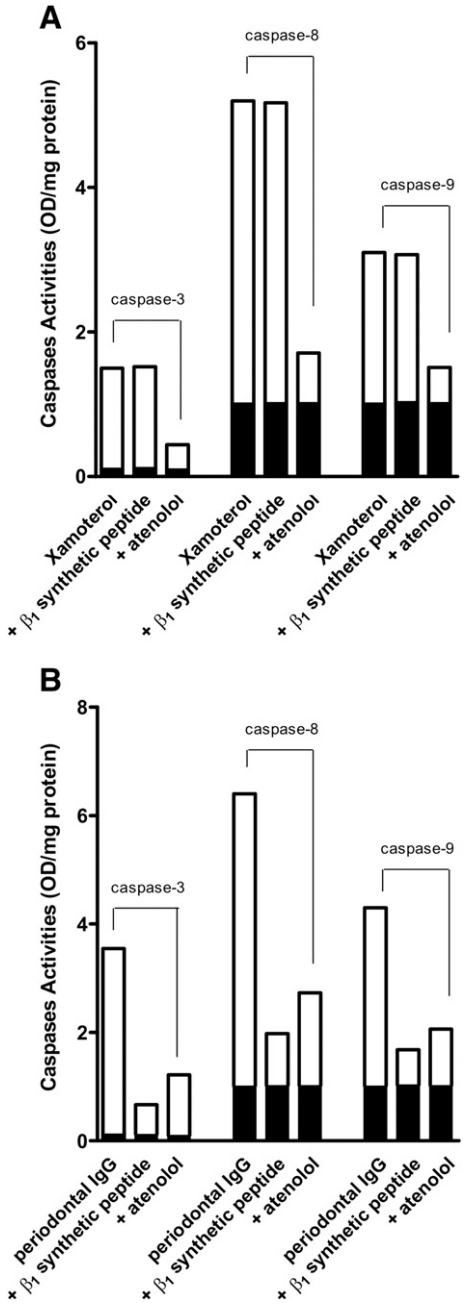

Fig. 7. A: Xamoterol alone (1×10^{-8} M) or in the presence of a β_1 synthetic peptide (5×10^{-5} M) or atenolol (1×10^{-7} M) (white column) on caspase-3, -8, and -9 activities in rat atria. Basal values (black column) are also shown. $P<0.0001$ between xamoterol alone versus xamoterol + atenolol. B: periodontal IgG alone (1×10^{-9} M) or in the presence of β_1 synthetic peptide (5×10^{-5} M) or atenolol (1×10^{-7} M) (white column) on caspase-3, -8, and -9 activities in rat atria. $P<0.0001$ between periodontal IgG alone versus periodontal IgG + atenolol and + β_1 synthetic peptide. Basal values (black column) are also shown. Six independent experiments were conducted in duplicate.

stimulation of NOS activity, cAMP and cGMP production, and PGE_2 generation was prevented by methylurea, ODQ (guanylate cyclase inhibitor), SQ22535, and INDO, respectively. Normal IgG had no effect in the system (Table 4).

4. Discussion

In the present study, we investigated the effects of anti-β_1-AR IgG from the sera of periodontitis patients and its role on atria β_1-AR-mediated regulation of cardiomyocyte apoptosis. We demonstrated that the antibody against β_1-AR is capable of activating the caspase (3, 9, and 8) pathway and also that the β_1 autoantibody has the ability to trigger increased production of NO and PGE_2. On the other hand, xamoterol, an authentic β_1-AR agonist, mimicked the effects of anti-β_1-AR IgG on the β_1-AR of isolated rat atria, apparently indicating the direct participation of atria β_1-AR in the apoptotic phenomenon, requiring not only the binding of β_1 autoantibody to the respective cardiac epitopes, but also the activation of atria β_1-AR. That a specific β_1 antagonist blocked the effects of both, xamoterol and the anti-β_1-AR antibody, confirmed this.

Moreover, the capacity of the β_1 synthetic peptide with an amino acid sequence identical to the second extracellular loop of human myocardial β_1-AR, able to absorb the effect of anti-β_1-AR IgG on atria β_1-AR, also supports the notion that cardiac β_1-AR antigens are essential for the functional activity of periodontal β_1 autoantibodies.

Recently, Sharma et al. [33,34] reported that metoprolol (β-AR antagonist) prevented the activation of cell death and attenuated cardiac dysfunction in diabetic heart, improving the cardiac output with no effect on the ejection fraction. Alternatively, metoprolol could prevent apoptosis through its action on β adrenergic signaling and its metabolic actions could decrease oxidative stress [35]. Moreover, three β-AR subtypes (β_1-AR, β_2-AR, β_3-AR) are expressed in cardiomyocytes and norepinephrine or isoproterenol stimulated all β-AR and induced apoptosis in rat cardiomyocytes and it is thought that a β_1-AR-mediated pathway primarily contributes to apoptosis [36,37].

The molecular mechanism of apoptosis involves the activation of caspases, a family of cysteine proteases that cleave intracellular target proteins at specific aspartate residues [38,39] and in mammals are grouped into two categories by their structure and function: “initiator caspases,” such as caspase-8 and -9 and “effector caspases,” such as caspase-3 [40]. Moreover, in apoptosis mitochondria release cytochrome *c* that, together with ATP, forms an activation complex that promotes caspase-9 activation [41] and the death receptor-mediated pathway involving tumor necrosis factor that interacts with caspase-8 [41]. Here, we detected cardiomyocyte apoptosis, showing that the periodontal anti-β_1-AR IgG, interacting with rat atria β_1-AR, has the capacity to increase active caspase-3, caspase-8, and caspase-9. This anti-β_1-AR antibody effect was also obtained with xamoterol, an authentic β_1-AR agonist, demonstrating that the β_1 autoantibody, via the activation of atria β_1-AR, provokes myocyte apoptosis. Both the anti-β_1-AR IgG and xamoterol effects on apoptosis were inhibited with the respective caspase-3, -8, and -9-specific peptides and by a β_1-AR antagonist, showing the participation of atria β_1-AR in the apoptotic phenomenon. The molecular mechanism by which anti-β_1-AR IgG provoked caspase activation involved several signaling pathways, including NOS activation, participation of calcium, PGE_2 generation, and JNK phosphorylation.

That an inhibitor of the iNOS isoform impaired the anti-β_1-AR IgG stimulating effect on caspase activities shows that NO has the capacity to induce cardiomyocyte apoptosis via the stimulation of cardiac soluble guanylate cyclase-dependent cGMP accumulation. This pro-apoptotic effect of NO has been reported previously; showing that NO caused apoptosis in macrophages [42], neurons [43], and vascular smooth muscle cells [44]. NO-mediated apoptosis is not completely understood [41] and NO-induced apoptosis often requires high levels of NO, a natural protective pathway leading to the activation of cardiac apoptotic phenomena [20,45].

Additionally, the anti-β_1-AR autoantibodies from the sera of periodontitis patients involve calcium mobilization and a cyclooxygenase (COX)-dependent mechanism in the activation of caspases. Moreover, xamoterol and anti-β_1-AR IgG were able to increase intracellular calcium concentrations and COX activity. The increase in intracellular calcium concentrations can regulate calcium influx and activate the CaM complex, because inhibitors of calcium influx (verapamil) and CaM complex (W-7) prevented the xamoterol and anti-β_1 autoantibody

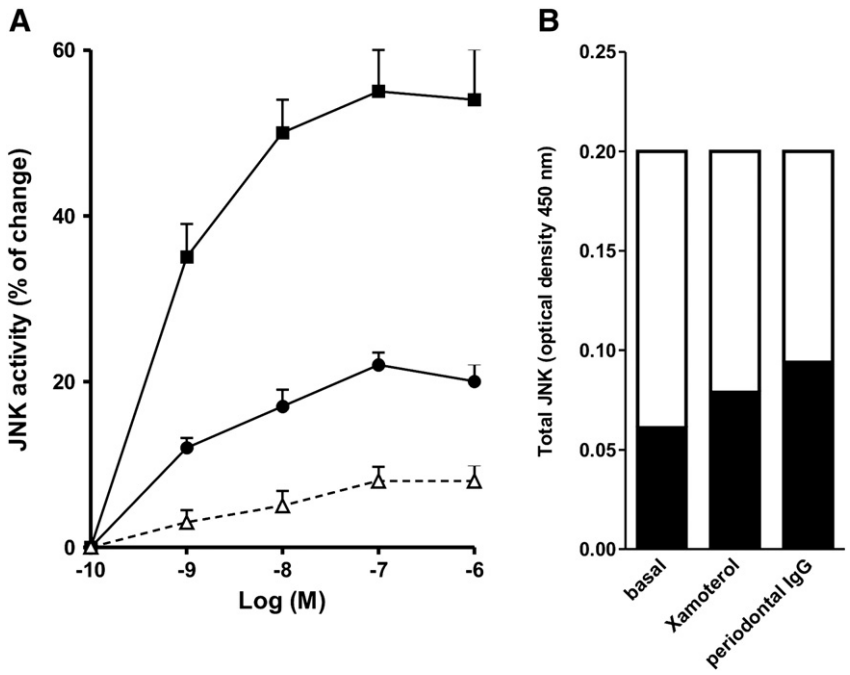

Fig. 8. A: Effect of periodontal IgG (■), xamoterol (●) and normal IgG (△) on JNK activity in rat atria. P<0.001 between normal IgG versus xamoterol and P<0.0001 between normal IgG versus periodontal IgG. B: Total JNK (black column) and JNK phosphorylated (white column). P<0.0001 between xamoterol alone or periodontal IgG alone versus basal. Each point represents the mean ± SE mean of six independent experiments, conducted in duplicate.

effect on caspases. On the other hand, the fact that INDO blocking rat atria COX activation not only blunted the effect of both xamoterol and anti-β_1-AR IgG on caspase activities, but also led to diminished PGE_2 production, pointing to the participation of PGE_2 in atria apoptosis through β_1 autoantibody-mediated caspase activation. In the present study, we showed that activation of β_1-AR in rat atria by anti-β_1-AR IgG increased PGE_2 generation. The increase in PGE_2 generation-mediated atria apoptosis is similar to that occurring in other tissues and was preceded by iNOS activation with an increase of NO [46–48]. The participation of PGE_2 in rat atria apoptosis shows that this cytokine

Table 3
Influence of different enzymatic inhibitors upon xamoterol and periodontal IgG on positive TUNEL nuclei.

Additions	Positive TUNEL nuclei (% of total)	*n*
No additions (basal)	10 ± 2	4
Xamoterol (1×10^{-8} M)	72 ± 6*	4
+ Z-DEVD-FMK (1×10^{-5} M)	36 ± 3**	4
+ Z-LEHD-FMK (1×10^{-5} M)	51 ± 4**	4
+ Z-IETD-FMK (1×10^{-5} M)	45 ± 3**	4
+ L-NMMA (1×10^{-5} M)	38 ± 3**	4
+ Methyl-Urea (5×10^{-5} M)	64 ± 5**	4
+ W-7 (5×10^{-5} M)	61 ± 4**	4
+ Verapamil (1×10^{-5} M)	60 ± 5**	4
+ BI-73D3 (1×10^{-4} M)	52 ± 4**	4
+ INDO (5×10^{-6} M)	59 ± 5**	5
+ SQ22535 (1×10^{-5} M)	56 ± 5**	5
Periodontal IgG (1×10^{-9} M)	78 ± 7*	5
+ Z-DEVD-FMK (1×10^{-5} M)	39 ± 4**	5
+ Z-LEHD-FMK (1×10^{-5} M)	52 ± 5**	5
+ Z-IETD-FMK (1×10^{-5} M)	55 ± 4**	5
+ L-NMMA (1×10^{-5} M)	49 ± 4**	4
+ Methyl-Urea (5×10^{-5} M)	55 ± 4**	4
+ W-7 (5×10^{-5} M)	54 ± 4**	4
+ Verapamil (1×10^{-5} M)	52 ± 5**	4
+ BI-73D3 (1×10^{-4} M)	47 ± 5**	4
+ INDO (5×10^{-6} M)	48 ± 4**	5
+ SQ22535 (1×10^{-5} M)	45 ± 5**	5
Normal IgG (1×10^{-9} M)	10 ± 3	4

Values are means ± SEM of n experiments in each group performed in duplicate. Basal values: no additions.
* P<0.0001, versus basal.
** P<0.0001, versus xamoterol or periodontal IgG.

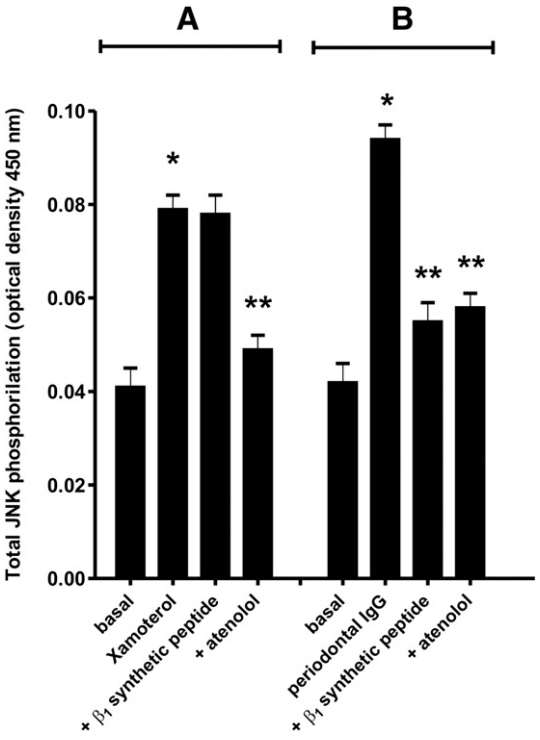

Fig. 9. A: Values of optical density of xamoterol 1×10^{-8} M alone or in the presence of a β_1 synthetic peptide 5×10^{-5} M or atenolol 1×10^{-7} M on rat atria JNK phosphorylation. B: Values of optical density of periodontal IgG 1×10^{-9} M alone or in the presence of a β_1 synthetic peptide 5×10^{-5} M or atenolol 1×10^{-7} M on rat atria JNK phosphorylation. Results are means ± SEM of nine independent patients in each group, performed in duplicate. *P<0.001, versus basal values; **P<0.0001, versus xamoterol or periodontal IgG alone.

Table 4
Effects of xamoterol, periodontal IgG, and enzyme inhibitors on NOS activity, cGMP, cAMP, and PGE_2 production in rat atria.

Additions	NOS	cGMP	cAMP	PGE_2
Basal	240 ± 18	58 ± 4	0.43 ± 0.03	18 ± 1.2
Xamoterol (1×10^{-8} M)	720 ± 62*	174 ± 12*	1.36 ± 0.10*	110 ± 10*
+ Methylurea (5×10^{-6} M)	576 ± 48**	–	–	–
+ ODQ (5×10^{-6} M)	–	136 ± 12**	–	–
+ SQ22535 (5×10^{-6} M)	–	–	1.12 ± .11**	–
+ INDO (5×10^{-5} M)	–	–	–	87 ± 8
Periodontal IgG (1×10^{-9} M)	680 ± 51*	175 ± 11*	1.29 ± 0.09*	108 ± 10*
+ Methylurea (5×10^{-6} M)	270 ± 8**	–	–	–
+ ODQ (5×10^{-6} M)	–	146 ± 13**	–	–
+ SQ22535 (5×10^{-6} M)	–	–	1.09 ± .10**	–
+ INDO (5×10^{-5} M)	–	–	–	77 ± 6**
Normal IgG (1×10^{-9} M)	238 ± 20	60 ± 5	0.45 ± 0.04	17 ± 2

Values are means ± SEM of seven experiments in each group performed in duplicate. Basal values: no additions. NOS, cGMP and cAMP were expressed as pmol/g tissue wet weight and PGE_2 was expressed as pg/mg tissue wet weight.
* $P<0.0001$, versus basal.
** $P<0.0001$, versus xamoterol and periodontal IgG.

is a modulator of the periodontal β_1 autoantibody stimulation/caspase effect. Moreover, when periodontal β_1 autoantibodies bind and activate rat atria β_1-AR, this leads to activation of adenylate cyclase, with an increase in cAMP production that, in turn, involves activation of PKA that ultimately modulates the heart apoptotic pathway. Similar observations have been made in neonatal rat ventricular myocytes and in the myocardium of rats and mice treated with isoproterenol [49,50].

Mitogen-activated protein kinases have important functions as mediators of intracellular signal transduction in the heart. Activation of JNK plays a pro-apoptotic role [51,52]. Our results show that the periodontal anti-β_1-AR IgG, or xamoterol, acting on atria β_1-AR increased JNK phosphorylation that, in turn, modulated the β_1 autoantibody effect on the caspases. There is a report suggesting that β_1-AR-stimulated apoptosis in rat ventricular myocytes [33] involves oxygen species/JNK-dependent activation of the mitochondrial death pathways and plays, in this way, a pro-apoptotic role.

Anti-β_1-AR autoantibodies from the sera of periodontitis patients play an important role in remodeling the rat myocardium. Rat atria β_1-AR, coupled with Gs/q, exerts a pro-apoptotic action via a cAMP-dependent mechanism, which appears to involve NOS activity, with an increase in NO levels, and cGMP, associated with activation of JNK, and increased generation of PGE_2. All of these events through the atria β_1-AR also involve activation of calcium channels and the CaM complex. These processes can shift the balance from apoptosis to myocardial cell survival during chronic β-AR stimulation and consequently, these events may have important clinical implications. As autoantibodies become fixed irreversibly to atria β_1-AR, they provoke structural heart changes that progressively alter the modulation of myocardial function, with possible progression to heart failure.

It is important to note, that the presence of serum antibodies to a particular periopathogen (*Porphyromonas gingivalis*, *Streptococcus pyogenes*, *Streptococcus mutans*) has been considered to reflect its involvement in the CP [53]. However, non-pathogenic periodontal bacteria can also elicit serum antibody response [54] but in our results, we hypothesize the possibility that periodontopathic bacteria may reflect a certain level of cross-reactivity between bacterial epitopes and heart β_1-AR epitopes; as a possible cause for the increased autoantibodies against β_1-AR in CP.

Further studies to determine the molecular mechanism by which interaction of β_1 autoantibodies present in the sera of periodontitis patients affects atria β_1-AR-stimulated apoptosis in cardiac myocytes, may have important implications in the link between cardiovascular disease and periodontitis. A proposed model to explain all of these apoptotic mechanisms whereby periodontal IgG and xamoterol acting on rat atria β_1-AR is shown in Fig. 10.

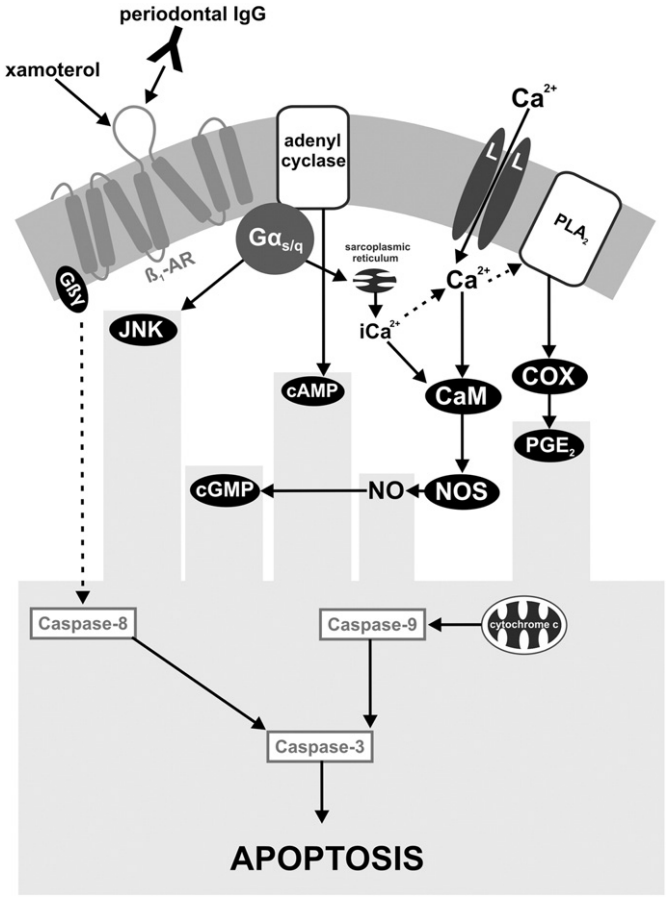

Fig. 10. Proposed model to explain the mechanism whereby periodontal IgG and xamoterol stimulate rat atria β_1-AR. Periodontal IgG and xamoterol both acting on atria β_1-AR activate the Gβγ subunit protein, leading to the activation of caspase-8 and the $G\alpha_{s/q}$ subunit, provoking JNK phosphorylation. Activation of adenylate cyclase leads to atrial cAMP accumulation with an increase in the efflux of extracellular calcium, which, in turn, increases intracellular calcium concentrations; calcium then binds to the calcium/calmodulin complex (CaM). The CaM complex increase nitric oxide synthase activity through the inducible isoform (iNOS) that, in turn, increases NO production, triggering cGMP accumulation. The rise in cytosolic calcium activates phospholipase A_2 (PLA_2) with activation of COX and PGE_2 generation. The activation of caspases-8 and -9 activates caspase-3, leading to rat atria apoptosis. The figure also shows, in shading, the modulation of JNK phosphorylation, the increase in cyclic nucleotides (cAMP and cGMP), and the production of nitric oxide (NO) and PGE_2, influencing the ability of caspases (3, 8, and 9) to generate the apoptotic phenomena by periodontal IgG and xamoterol in rat atria. Filled arrows indicate direct mechanisms and dotted arrows indicate indirect mechanisms.

Acknowledgments

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References

[1] Iwata M, Yoshikawa T, Baba A, Anzai T, Mitamura H, Ogawa S. Autoantibodies against the second extracellular loop of beta1-adrenergic receptors predict ventricular tachycardia and sudden death in patients with idiopathic dilated cardiomyopathy. J Am Coll Cardiol 2001;37:418–24.
[2] Limas CJ, Goldenberg IF, Limas C. Autoantibodies against β-adrenoceptors in human idiopathic dilated cardiomyopathy. Circ Res 1989;64:97–103.
[3] Sterin-Borda L, Perez Leiros C, Wald M, Cremaschi G, Borda E. Antibodies to beta 1 and beta 2 adrenoreceptors in Chagas' disease. Clin Exp Immunol 1988;74:349–54.
[4] Sterin-Borda L, Gorelik G, Genaro A, Goin JC, Borda ES. Human chagasic IgG interacting with lymphocyte neurotransmitter receptors triggers intracellular signal transduction. FASEB J 1990;4:1661–7.

[5] Segovia M, Ganzinelli S, Reina S, Borda E, Sterin-Borda L. Role of anti-β(1)adrenergic antibodies from patients with periodontitis in cardiac dysfunction. J Oral Pathol Med 2012;41:242–8.
[6] Segovia M, Reina S, Borda E, Sterin-Borda L. Autoantibodies to the β(1)-adrenoceptor from patients with periodontitis as a risk factor for cardiac dysfunction. ISRN Dent 2011:791393.
[7] Jahns R, Boivin V, Hein L, Triebel S, Angermann CE, Ertl G, et al. Direct evidence for a beta 1-adrenergic receptor-directed autoimmune attack as a cause of idiopathic dilated cardiomyopathy. J Clin Invest 2004;113:1419–29.
[8] Jahns R, Boivin V, Siegmund C, Inselmann G, Lohse MJ, Boege F. Autoantibodies activating human beta1-adrenergic receptors are associated with reduced cardiac function in chronic heart failure. Circulation 1999;99:649–54.
[9] Freedman NJ, Lefkowitz RJ. Anti-beta(1)-adrenergic receptor antibodies and heart failure: causation, not just correlation. J Clin Invest 2004;113:1379–82.
[10] Sterin-Borda L, Gorelik G, Postan M, Gonzalez Cappa S, Borda E. Alterations in cardiac beta-adrenergic receptors in chagasic mice and their association with circulating beta-adrenoceptor-related autoantibodies. Cardiovasc Res 1999;41: 116–25.
[11] Borda ES, Sterin-Borda L. Antiadrenergic and muscarinic receptor antibodies in Chagas' cardiomyopathy. Int J Cardiol 1996;54:149–56.
[12] Liu J, Mao W, Iwai C, Fukuoka S, Vulapalli R, Huang H, et al. Adoptive passive transfer of rabbit beta1-adrenoceptor peptide immune cardiomyopathy into the Rag2−/− mouse: participation of the ER stress. J Mol Cell Cardiol 2008;44: 304–14.
[13] Sterin-Borda L, Cantore M, Pascual J, Borda E, Cossio P, Arana R, et al. Chagasic IgG binds and interacts with cardiac beta adrenoceptor-coupled adenylate cyclase system. Int J Immunopharmacol 1986;8:581–8.
[14] Mao W, Fukuoka S, Iwai C, Liu J, Sharma VK, Sheu SS, et al. Cardiomyocyte apoptosis in autoimmune cardiomyopathy: mediated via endoplasmic reticulum stress and exaggerated by norepinephrine. Am J Physiol Heart Circ Physiol 2007;293: H1636–45.
[15] Zhu WZ, Wang SQ, Chakir K, Yang D, Zhang T, Brown JH, et al. Linkage of beta1-adrenergic stimulation to apoptotic heart cell death through protein kinase A-independent activation of Ca2+/calmodulin kinase II. J Clin Invest 2003;111: 617–25.
[16] Joensen L, Borda E, Kohout T, Perry S, García G, Sterin-Borda L. Trypanosoma cruzi antigen that interacts with the beta1-adrenergic receptor and modifies myocardial contractile activity. Mol Biochem Parasitol 2003;127:169–77.
[17] Ishikawa Y, Homcy CJ. The adenylyl cyclases as integrators of transmembrane signal transduction. Circ Res 1997;80:297-04.
[18] Communal C, Singh K, Pimentel DR, Colucci WS. Norepinephrine stimulates apoptosis in adult rat ventricular myocytes by activation of the beta-adrenergic pathway. Circulation 1998;98:1329–34.
[19] Tomita H, Nazmy M, Kajimoto K, Yehia G, Molina CA, Sadoshima J. Inducible cAMP early repressor (ICER) is a negative-feedback regulator of cardiac hypertrophy and an important mediator of cardiac myocyte apoptosis in response to beta-adrenergic receptor stimulation. Circ Res 2003;93:12–22.
[20] Wang Y, Chen C, Loake G, Chu C. Nitric oxide: promoter or suppressor of programmed cell death? Protein Cell 2010;1:133-42.
[21] Crow MT, Mani K, Nam YJ, Kitsis RN. The mitochondrial death pathway and cardiac myocyte apoptosis. Circ Res 2004;95:957–70.
[22] Adams JM, Cory S. Life-or-death decisions by the Bcl-2 protein family. Trends Biochem Sci 2001;26:61–6.
[23] Masri C, Chandrashekhar Y. Apoptosis: a potentially reversible, meta-stable state of the heart. Heart Fail Rev 2008;13:175–9.
[24] Andriankaja OM, Genco RJ, Dorn J, Dmochowski J, Hovey K, Falkner KL, et al. Periodontal disease and risk of myocardial infarction: the role of gender and smoking. Eur J Epidemiol 2007;22:699–705.
[25] Cairo F, Castellani S, Gori AM, Nieri M, Baldelli G, Abbate R, et al. Severe periodontitis in young adults is associated with sub-clinical atherosclerosis. J Clin Periodontol 2008;35:465–72.
[26] Humphrey LL, Fu R, Buckley DI, Freeman M, Helfand M. Periodontal disease and coronary heart disease incidence: a systematic review and meta-analysis. J Gen Intern Med 2008;23:2079–86.
[27] Espinola-Klein C, Rupprecht HJ, Blankenberg S, Bickel C, Kopp H, Rippin G, et al. Impact of infectious burden on extent and long-term prognosis of atherosclerosis. Circulation 2002;105:15–21.
[28] Desvarieux M, Demmer RT, Rundek T, Boden-Albala B, Jacobs Jr DR, Sacco RL, et al. Periodontal microbiota and carotid intima–media thickness: the Oral Infections and Vascular Disease Epidemiology Study (INVEST). Circulation 2005;111: 576–82.
[29] Page RC, Eke PI. Case definitions for use in population-based surveillance of periodontitis. J Periodontol 2007;78:1387–99.
[30] Sterin-Borda L, Gorelik G, Borda ES. Chagasic IgG binding with cardiac muscarinic cholinergic receptors modifies cholinergic-mediated cellular transmembrane signals. Clin Immunol Immunopathol 1991;61:37–97.
[31] Parlakpinar H, Sahna E, Acet A, Mizrak B, Polat A. Protective effect of caffeic acid phenethyl ester (CAPE) on myocardial ischemia–reperfusion-induced apoptotic cell death. Toxicology 2005;209:1–14.
[32] Wylie PG, Challiss RA, Blank JL. Regulation of extracellular-signal regulated kinase and c-Jun N-terminal kinase by G-protein-linked muscarinic acetylcholine receptors. Biochem J 1999;338:619–28.
[33] Sharma V, Parsons H, Allard MF, McNeill JH. Metoprolol increases the expression of beta(3)-adrenoceptors in the diabetic heart: effects on nitric oxide signaling and forkhead transcription factor-3. Eur J Pharmacol 2008;595:44–51.
[34] Sharma V, Dhillon P, Wambolt R, Parsons H, Brownsey R, Allard MF, et al. Metoprolol improves cardiac function and modulates cardiac metabolism in the streptozotocin-diabetic rat. Am J Physiol Heart Circ Physiol 2008;294:H1609–20.
[35] Sharma V, Sharma A, Saran V, Bernatchez PN, Allard MF, McNeill JH. β-receptor antagonist treatment prevents activation of cell death signaling in the diabetic heart independent of its metabolic actions. Eur J Pharmacol 2011;657:117-25.
[36] Bristow MR, Hershberger RE, Port JD, Gilbert EM, Sandoval A, Rasmussen R, et al. Beta-adrenergic pathways in non failing and failing human ventricular myocardium. Circulation 1990;82:I12–25.
[37] Jane-wit D, Altuntas CZ, Johnson JM, Yong S, Wickley PJ, Clark P, et al. Beta 1-adrenergic receptor autoantibodies mediate dilated cardiomyopathy by agonistically inducing cardiomyocyte apoptosis. Circulation 2007;116:399-410.
[38] Nicholson DW, Thornberry NA. Caspases: killer proteases. Trends Biochem Sci 1997;22:299-306.
[39] Thornberry NA, Lazebnik Y. Caspases: enemies within. Science 1998;281:1312–6.
[40] Liu X, Kim CN, Yang J, Jemmerson R, Wang X. Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome c. Cell 1996;86:147–57.
[41] Ekhterae D, Lin Z, Lundberg MS, Crow MT, Brosius III FC, Nunez G. ARC inhibits cytochrome c release from mitochondria and protects against hypoxia-induced apoptosis in heart-derived H9c2 cells. Circ Res 1999;85:e70–7.
[42] Albina JE, Cui S, Mateo RB, Reichner JS. Nitric oxide-mediated apoptosis in murine peritoneal macrophages. J Immunol 1993;150:5080–5.
[43] Heneka MT, Loschmann PA, Gleichmann M, Weller M, Schulz JB, Wullner U, et al. Induction of nitric oxide synthase and nitric oxide-mediated apoptosis in neuronal PC12 cells after stimulation with tumor necrosis factor-alpha/lipopolysaccharide. J Neurochem 1998;71:88–94.
[44] Lincoln TM, Cornwell TL, Komalavilas P, Boerth N. Cyclic GMP-dependent protein kinase in nitric oxide signaling. Methods Enzymol 1996;269:149–66.
[45] Kim Y-M, Bombeck CA, Billiar TR. Nitric oxide as a bifunctional regulator of apoptosis. Circ Res 1999;84:253–6.
[46] Maher TM, Evans IC, Bottoms SE, Mercer PF, Thorley AJ, Nicholson AG, et al. Diminished prostaglandin E2 contributes to the apoptosis paradox in idiopathic pulmonary fibrosis. Am J Respir Crit Care Med 2010;182:73–82.
[47] Takadera T, Shiraishi Y, Ohyashiki T. Prostaglandin E2 induced caspase-dependent apoptosis possibly through activation of EP2 receptors in cultured hippocampal neurons. Neurochem Int 2004;45:713–9.
[48] Magari M, Nishikawa Y, Fujii Y, Nishio Y, Watanabe K, Fujiwara M, et al. IL-21-dependent B cell death driven by prostaglandin E2, a product secreted from follicular dendritic cells. J Immunol 2011;187:4210–8.
[49] Singh K, Xiao L, Remondino A, Sawyer DB, Colucci WS. Adrenergic regulation of cardiac myocyte apoptosis. J Cell Physiol 2001;189:57–65.
[50] Krishnamurthy P, Subramanian V, Singh M, Singh K. Beta1 integrins modulate beta-adrenergic receptor-stimulated cardiac myocyte apoptosis and myocardial remodeling. Hypertension 2007;49:865–72.
[51] Menon B, Johnson JN, Ross RS, Singh M, Singh K. Glycogen synthase kinase-3beta plays a pro-apoptotic role in beta-adrenergic receptor-stimulated apoptosis in adult rat ventricular myocytes: role of beta1 integrins. J Mol Cell Cardiol 2007;42: 653–61.
[52] Singh M, Roginskaya M, Dalal S, Menon B, Kaverina E, Boluyt MO, et al. Extracellular ubiquitin inhibits beta-AR-stimulated apoptosis in cardiac myocytes: role of GSK-3beta and mitochondrial pathways. Cardiovasc Res 2010;86:20–8.
[53] Haffajee AD, Socransky SS. Microbial etiological agents of destructive periodontal diseases. Periodontol 2000 1994;5:78–111.
[54] Ebersole JL, Taubman MA, Smith DJ, Frey DE, Haffajee AD, Socransky SS. The relationship of antibody response categories to clinical parameters of periodontal disease. J Periodontal Res 1984;19:609–13.