Adaptação de um sistema de ensaio biológico para detecção de fatores relaxantes endoteliais derivados do endocárdio atrial canino

Adaptation of bioassay to detect endothelium-derived relaxing factors from the canine atrial endocardium

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Resumo

Objetivo: **Estudar a liberação de fatores relaxantes derivados do endotélio (EDRF) pelo endocárdio de aurículas de corações caninos.**

Métodos: **Aurículas atriais caninas foram suturadas em forma de tubos e o efluente desses tubos foram submetidos a ensaios biológicos (sistema de perfusão isolada em câmaras de órgãos) utilizando artéria coronária canina, para a detecção de EDRFs.**

Resultados: **O efluente da aurícula direita promoveu relaxamento de 58,4 $\pm$ 10,1% e da aurícula esquerda 74,9 $\pm$ 8,5% da contração inicial obtida pela ação da prostagladina F2α em artéria coronária. Não houve diferença estatística no relaxamento da artéria coronária induzido pelos efluentes das aurículas direita e esquerda. O relaxamento induzido pelos efluentes das aurículas direita e esquerda foi abolido pelo tratamento das mesmas com Triton X-100. O tratamento das aurículas com L-NMMA, um inibidor competitivo da síntese de óxido nítrico, e com indometacina, um inibidor da via da ciclooxigenase, promoveu redução no relaxamento da artéria coronária induzido pelo efluente auricular, indicando que o endotélio endocárdico libera óxido nítrico e prostanóides.**

Conclusões: **Esse estudo demonstra, pela primeira vez, a liberação luminal *in vitro* de EDRF e prostaciclina pelo átrio de coração canino. A habilidade do endotélio endocárdico em produzir esses fatores pode ter um papel importante na prevenção da formação de trombos nas câmaras cardíacas.**

Descritores: **Átrios do coração. Endotélio. Óxido nítrico. Endocárdio.**

Abstract

Objective: **The aim of this study was to assess the release of endothelium-derived relaxing factors from the endocardium of canine atrial appendage.**

Methods: **To study the release of endothelium-derived**

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relaxing factor (EDRF) from intact atrial endocardial endothelium, tube-shaped sutures of canine atrial appendages were performed and effluents from these tubes were bioassayed (isolated perfused organ chamber system) for detection of EDRF in canine coronary artery.

Results: Effluent from the right atrial appendage caused a relaxation of 58.4 $\pm$ 10.1% and the left atrial appendage 74.9 $\pm$ 8.5% from the initial prostagladin F2α contraction in coronary artery. No significant statistical difference was detected in effluent from the right and left atrial appendages. This relaxation was abolished by treating the heart tubes with Triton X-100 and reduced by treatment with LNMMA, a competitive inhibitor of nitric oxide and with indomethacin, an inhibitor of the cyclo-oxygenase pathway, also indicating the release of vasodilatory prostanoids from the endocardial endothelium.

Conclusion: This study showed for the first time, *in vitro* luminal release of EDRF and prostacyclin from the canine heart atrium. The ability of the endocardial endothelium to produce these factors could play an important role in preventing thrombus formation in the cardiac chambers.

Descriptors: Heart atria. Endothelium. Nitric oxide. Endocardium.

INTRODUCTION

The endocardial endothelium (EE) is a monolayer cellular that covers internally the heart. The endocardial endothelial cells (EECs) also constitute a very large contact surface area that offers a very high ratio of cavity surface area to atrial volume, a finding that suggests an important sensory role for the EE. These cells play the role of a physic-chemical barrier between the cardiomyocytes and the circulating blood [1-3]. Furthermore, just like other types of endothelial cells, EECs release several factors such as nitric oxide (NO), angiotensin II [3], endothelin and prostacyclin [4,5].

Although endocardial endothelium capillary and myocardial endothelium share common features in their effects on subjacent cardiomyocytes, there is growing evidence that these two cardiac endothelial cell types are not identical. They differ with regard to developmental, morphological and functional properties, the major difference probably resulting from their different position and contribution within the overall endothelial system [1,2].

Cultured porcine endocardial endothelium has been shown to produce an unstable humoral factor whose effects are indistinguishable from endothelium-derived relaxing factor (EDRF) [6]. Canine cardiac valve endothelium has also been shown to release EDRF and vasodilator prostanoids [7]. The active component of EDRF is the nitric oxide radical [8,9], which is also the active component of nitro-vasodilators such as sodium nitroprusside and nitroglycerin. Both EDRF and prostacyclin (PGI$_2$) have been shown to inhibit platelet aggregation and adhesion [10] in addition to their important vasodilator effects [11]. The ability of the endocardial endothelium to produce these antithrombotic factors plays an important role in preventing thrombus formation in the cardiac chambers [12].

Previous experiments about endocardium function, have utilized endocardial endothelial cells culture isolated from ventricles, but this method presents problems as a down-regulation of receptors when the cells are cultured and the response of the cultured endothelium to agonists can be altered [13,14]. In the present experiments, a bioassay technique was developed to detect luminal release of endothelium-derived relaxing factors from intact canine atrial endocardium.

METHODS

Bioassay technique

Heartworm free mongrel dogs (25-30 kg; n=10) of either sex were anesthetized with intravenous pentobarbital sodium (30 mg/kg bolus injection; Fort Dodge Laboratories) and exsanguinated via the carotid arteries; the beating heart was excised and immersed in cool, oxygenated physiological salt solution of the following millimolar composition: NaCl, 118.3; KCl, 4.7; MgSO$_4$, 1.2; KH$_2$PO$_4$, 1.22; CaCl$_2$, 2.5; NaHCO$_3$, 25.0 and glucose, 11.1 (control solution). The procedures and the handling of the animals were reviewed and approved by the Institutional Animal Care and Use Committee of the Mayo Foundation.

The left circumflex coronary artery was carefully dissected free from the heart and placed in control solution. A coronary ring of about 3 mm was cut and the endothelium removed with a pair of watchmakers forceps. This procedure effectively removes endothelium from the coronary ring without affecting the smooth muscle ability to contract or relax. Both the right and left atrial appendages were removed as close to its base as possible, and placed in physiological salt solution. The atrium is then sutured into a tube with two layers of continuous 5/O prolene and its ends tied to the bioassay lines. The setup is then checked for leaks and repaired when necessary. The whole suturing process which takes about 10 minutes, was done in a dish containing the control solution and the tissue was kept under solution as much as possible to prevent damage to the endothelium (Figure 1).
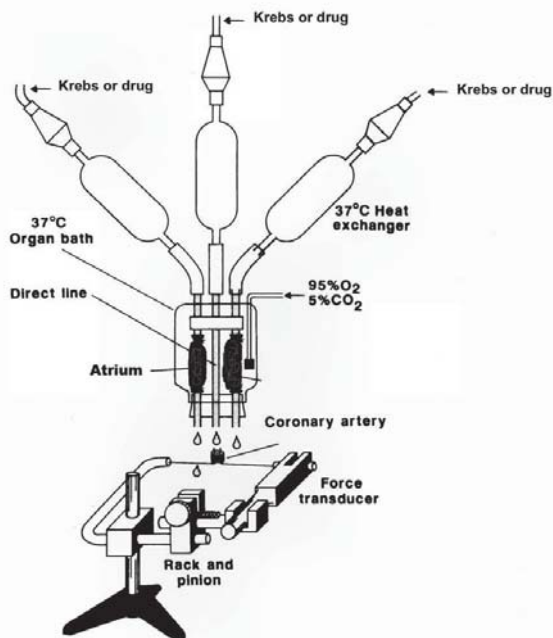
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Fig. 1 - Bioassay system from the perfused atrial tube. The atriuns are perfused through a separate constant-flow perfusion loop, and vasoactivity of effluent from the atriuns was bioassayed on a ring of canine coronary artery. The central cannula was used to direct perfusion of coronary rings (physiologic solution with or without drugs). The laterals cannulas was connected to the right and left atrium and the solution perfused thought atrial tubes was dripped on coronary ring. The coronary ring was connected to a force transducer that registers the variation of vascular tone (contraction and relaxation). Modified from Rubanyi et al., 1985 [44]

In this system, the biological activity of endothelium-derived relaxing factor released from the atrial tube was bioassayed by a coronary ring (proximal left circumflex coronary artery) from which the endothelium has been removed. The atrial tubes were perfused at a constant and flow (5 ml min^{-1}) with physiological solution at 37°C and bubbled with 95% oxygen. There was a transient delay of one second before the fluid reached the bioassay coronary rings, which were suspended below the donor segment. The tension developed in the rings was recorded. The bioassay coronary rings were first superfused for 60 minutes with control solution, which passed through a stainless steel cannula (direct superfusion). During this time, coronary rings were stretched in a stepwise fashion to their optimal tension (10g) (Figure 1).

Relaxation response of the bioassay coronary rings, were examined during a contraction caused by prostaglandin F_{2a} (PGF$_{2a}$, 2x10^{-6}M). The absence of endothelium of the bioassay coronary rings was confirmed by the lack of relaxation to calcium ionophore A23187 (10^{-6}M) infused

under direct superfusion. The bioasssay coronary rings could also be superfused with effluent from the atrial tubes. After a period of stabilisation with superfusion from the atrial effluent, calcium ionophore A23187 (10^{-6}M) was added to solution that perfuse the atrium tube and efflux to coronary ring. In some experiments, after stimulation of the atrium with calcium ionophore, indomethacin (10^{-5}M) and N^{G}-monomethyl-L-arginine (LNMMA, 10^{-4}M) is then added to the superfusion solution (Figure 2A). When indomethacin or nitric oxide inhibitors are used, the atrial tissue was allowed to be treated for 45 to 60 minutes before relaxation responses were tested again.

In some experiments, the atrial endocardial endothelium was removed by infusing one ml of 1% Triton X-100 through the atrial tubes. After infusion of the Triton X-100, the atrial tubes were immediately flushed with at least 500 ml of warm physiological solution. This procedure was done without removing the atrial tubes from the bioassay system and great care is taken to ensure that the Triton X-100 did not come into contact with the bioassay coronary rings (Figure 2B). The coronary rings were then superfused with PGF$_{2a}$ for at least 30 minutes before relaxation response from the atrial effluent to be tested again. At the end of experiment after Triton X-100 was used, the bioassay coronary rings were tested with sodium nitroprusside (10^{-4}M) which was infused through direct superfusion to ensure that the bioassay coronary rings was not damage by Triton X-100.
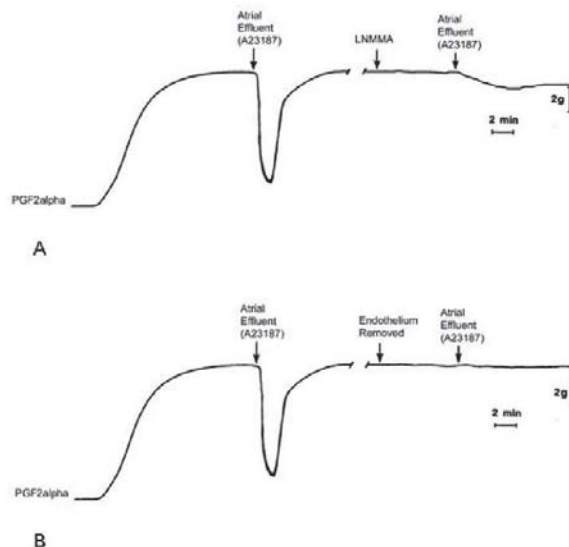

Fig. 2 - Representative recording of change in contraction of canine coronary artery ring with endothelium superfused with effluent from atrial tubes infused with A23187. Relaxation was inhibit when the ring was superfused with L-NMMA (A) and when the endothelium was removed (B)

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Drugs

The following drugs were used: acetylcholine, indomethacin, prostagladin $F_{2\alpha}$, calcium ionophore A23187 and Triton X-100 (all from Sigma Chemical Company, St Louis, MO). N^G-monomethyl-L-arginine were obtained from Calbiochem, San Diego, CA. Triton X-100 was diluted with physiological control solution to make a final concentration of 1%. All powdered drugs were prepared daily with distilled water except for indomethacin which was prepared with Na_2CO_3 (10^{-5}M).

Data analysis

The results were expressed as means $\pm$ SEM. For each experiment the number of the animals was 10. Relaxations are expressed as percent change in tension from the contraction of the bioassay ring to prostaglandin $F_{2\alpha}$. Statistical evaluation of the data was performed by Two-way ANOVA and Bonferroni post-test. Values were considered to be significantly different when *P* was smaller than 0.05.

RESULTS

The physiologic solution with $PGF_{2\alpha}$ ($2x10^{-6}$M) infused through the atrial tubes induced a stable contraction in the canine coronary artery bioassay ring of 6.54 $\pm$ 0.61. During contraction with $PGF_{2\alpha}$, the superfusion of the bioassay ring was switched from the direct line to superfusion from the atrium (endocardial superfusion). There was no change in tension of the contracted bioassay ring when the superfusion was switched from the direct line to the endocardial perfusate. However, when the calcium ionophore A23187 was infused into the atrial tubes, the bioassay ring exhibited a relaxation of 58.4 $\pm$ 10.1% from right atrium and 74.9 $\pm$ 8.5% from left atrium of the initial prostaglandin $F_{2\alpha}$ contraction. There was no significant difference between the magnitude of vasodilation induced by the effluent from the right versus left atrium (*P* > 0.05).

The vasodilator activity of effluent from the right and left atrium was blocked 85.1 $\pm$ 7.9% and 88.9 $\pm$ 3.5%, respectively by the presence of the L-NMMA (10^{-4}M) in the perfusion fluid, indicating that endocardium-derived NO are totally involved in producing the relaxation of the bioassay ring (Figure 3). In contrast, indomethacin blocked 67.5 $\pm$ 5.2% of the production of vasodilator by the perfused right atrium and 68.8 $\pm$ 6.9% by the perfused left atrium, indicating that endocardium-derived prostanoids are partially involved in producing the relaxation of the bioassay ring (Figure 4). The treatment of the atrium with Triton X-100 1% completely blocked the relaxation induced by A23187 infusion in coronary artery (Figure 5).
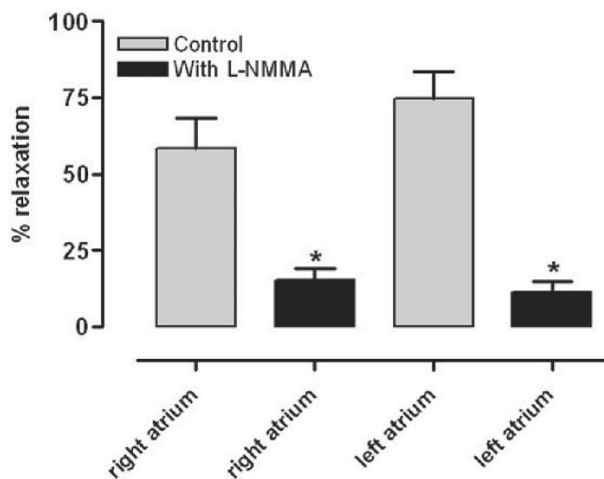

*Fig. 3 - Bioassay of calcium ionophore-induced promote relaxation in the right and left atrium. The vasodilator activity of effluent from the right and left atrium was blocked 85.1 $\pm$ 7.9% and 88.9 $\pm$ 3.5%, respectively by the presence of the L-NMMA (10^{-4}M) in rings contracted with $PGF_{2\alpha}$ ($2x10^{-6}$M). Results are reported as means $\pm$ SEM. * indicate significant difference between with and without L-NMMA groups (P < 0,001)*

*Fig. 4 - Bioassay of calcium ionophore-induced promote relaxation in the right and left atrium. The vasodilator activity of effluent from the right and left atrium was blocked blocked 67.5 $\pm$ 5.2% and 68.8 $\pm$ 6.9% respectively by the presence of the indomethacin (10^{-5}M) in rings contracted with $PGF_{2\alpha}$ ($2x10^{-6}$M). Results are reported as means $\pm$ SEM. * indicate significant difference between with and without indomethacin groups (P < 0,001)*

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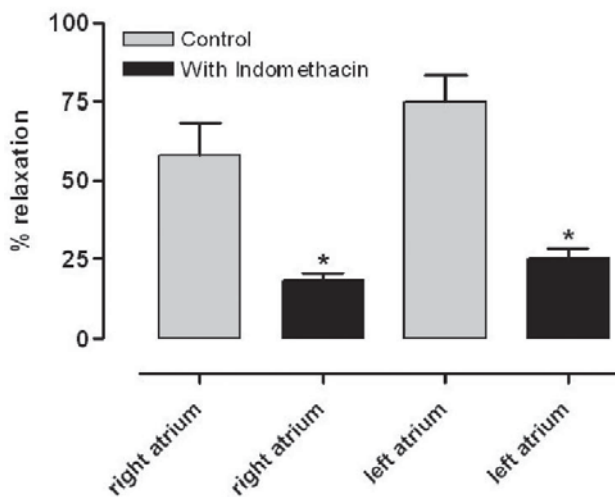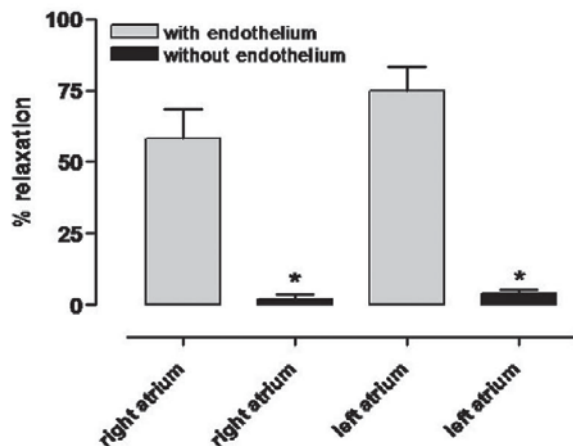
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*Fig. 5 - Bioassay of calcium ionophore-induced promote relaxation in the right and left atrium. The vasodilator activity of effluent from the right and left atrium was blocked 97.1 + 2.9% and 95.9 + 2.5%, respectively by endothelium removal in rings contracted with PGF2α (2x10-6M). Results are reported as means ± SEM. * indicate significant difference between with and without endothelium ring (P < 0,001)*

DISCUSSION

This study showed the luminal release of Endothelium-derived nitric oxide (EDNO) and prostacyclin (PGI_2) from intact atrial EE. Both, right and left atrium produced vasodilating substance in quantities sufficient to induce relaxation of coronary artery smooth muscle, which was used as a detector tissue. Previous studies on the release of EDNO and PGI_2 have utilized cultured EE and cardiac valve leaflets. Cultured endothelial cells have shown changes in response to agonists probably as a result of receptor down-regulation. Therefore, the bioassay can be more effective for showing the presence and functionality of these vasodilators.

The vasodilatation induced by stimulating with calcium ionophore A23187 was complete abolished after treatment with Triton X-100, confirming the origin of the relaxing factors from the endothelium. It is important to report that before choosing the calcium ionophore A23187 as a pharmacological tool for this study, we used acetylcholine as agonist. Unfortunately, acetylcholine was unable to induce relaxing factor release from canine endocardium endothelium. We could not find reports, in the literature, about muscarinic agonist-induced relaxing factors release from canine endocardium endothelium. Considering data obtained from fish [15] and frog [16], in which acetylcholine induced NO release from endocardium, we can hypothesized that muscarinic-receptor distribution on EE can be specie-dependent. In addition, using the same bioassay principle, we had to choose calcium ionophore A23187 to promote human saphenous vein NO release [17,18] or to bioassay endocardial NO production in the beating [19].

The vasodilatation was also significantly reduced by treatment with L-NMMA which is a competitive inhibitor of nitric oxide synthesis from L-arginine [20-22] and with indomethacin, cyclo-oxygenase inhibitor [23]. Thus it seems both EDNO and PGI_2 are produced from the atrium and are as effective as the vascular. This finding supports the data obtained in the experiments using this technique on beating heart, showing that vasodilator produced by the intact heart endocardium is NO-dependent as evidenced with the complete inhibition of endocardium-dependent relaxing activity by the administration of NG-nitro-L-arginine. In Discigil et al. [19] investigation, isolated rabbit hearts were perfused retrograde in the aortic root with a balanced salt solution using a Langendorff technique. To detect endocardial vasodilator production, right heart perfusate was used to bathe a "bioassay" segment of canine coronary artery denuded of endothelium. As observed on the present protocol, calcium inophore produced relaxation of the bioassay smooth muscle when added to the cardiac perfusate, but not when applied directly to the bioassay segment.

The vasodilator effect of A23187 was reduced 70% and 85% after treatment with indomethacin and L-NMMA, respectively. Since we don't performed experiments with the combination of L-NMMA and Indomethacin, the inhibition less than 100% in both can be explained by NO or PGI_2 participation (remembering that the NOS or COX inhibition was not simultaneous), endothelial-derived hyperpolarizing factor participation, or a cross talk between NO and PGI_2 production. In endothelial [24] and muscular [25] culture cell, the PGI_2 production was significantly enhanced by L-NAME incubation and the NO production was also enhanced after COX inhibition, although to a lesser degree, showing that exist a cross talk between NO and PGI_2 [24-27]. This can be important to maintain the vascular homeostasis in pathological situations.

The NO and the PGI_2 have been shown to inhibit platelet aggregation and adhesion [10] in addition to their important vasodilator effects [11]. Activation of the formation of cyclic AMP in vascular smooth muscle during relaxations evoked by PGI_2, augments the responsiveness to EDRF. Furthermore, PGI_2 also stimulates the release of EDRF [28]. In addition, NO which accounts for the actions of EDRF [29], potentiates anti-aggregatory properties of PGI_2; they both can prevent platelet aggregation together at

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concentrations which on their own too low to inhibit aggregation [30,31]. NO also inhibits platelet adhesion to the vascular endothelium which contributes to non-thrombogenicity of endothelial cell [32-34]. The ability of the EE to produce these antithrombotic factors plays an important role in preventing thrombus formation in the cardiac chambers [30,35]. The NO also are involved on atrial fibrillation associated with endothelial dysfunction [36].

Nitric oxide production by vascular endothelium is an important mechanism of vascular tone and platelet aggregation in mammals [37]. Indeed endothelium-dependent vasodilation is present even in vasculature of lower vertebrates [38]. Thus NO radical production by some vascular tissue functions more as an anti-thrombotic agent than a vasodilator.

In the present model, we could not demonstrate a basal release of EDNO (i.e. release in the absence of stimulation by an agonist from the intact atrium, as is typically present in other vessels [39]. The passage of blood in the vessels generates hemodynamic forces and regulates the function of endothelial cells lining the intimal surface of the vasculature [40]. Shear stress stimulates the synthesis and secretion of various bioactive molecules such as PGI_2 and NO. This basal release of NO and PGI_2 promoted by physiological shear stress flow, may not have been demonstrated because the flow (5 ml min^{-1}) applied to the perfusate could not be optimum to promote shear stress and induce basal release of this factors. However, the need to handle the atrium by suturing it into a tube and the high partial pressure of oxygen of the perfusate utilized in this experiment could have generated oxygen-riched radicals which inactivates nitric oxide [41], justifying the relaxation found less than 100% in both left and rigth atrium by A23187 and absence of relaxation from basal release of EDNO.

Bioassay vascular endothelium substances became mandatory since Furchgott and Zawadzki [42,43] proposed the existence of EDRF, including the studies about the basal release of this factor. Our first motivation was based on two capitol studies: the setup used by Rubanyi et al. [44], at Mayo Clinic and the Brutsaert et al. studies and theories about the endocardium endothelium [1-3]. In other words, we had as research targets to setup methods to bioassay endocadium-derived relaxing factor(s).

In conclusion, this experiment demonstrated, for the first time, luminal release of endothelium-derived NO and PGI_2 from the intact canine atrium. EDNO and PGI_2 may act the synergic way [30] as important anti-thrombotic factor in the atrium. Considering that cultured endothelial cells have shown changes in response to agonists as a result of receptor down-regulation [13,14], the proposed methodology can be more useful due to its easy execution, low cost, good reproducibility and effectiveness in mimicking in vivo conditions.

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