α-adrenoceptor-mediated enhanced inducibility of atrial fibrillation in a canine system inflammation model

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Abstract. The exact mechanism associated with inflammation and atrial fibrillation (AF) remains unknown. The aim of the present study was to investigate the roles of connexin 43 (Cx43) and α1-adrenergic receptor (α1-AR) activation in the pathogenesis of system inflammation-induced AF. A canine model of chronic low-grade system inflammation was established by administrating a low dose of lipopolysaccharide (LPS; 0.1 µg/kg) for 2 weeks. Programmed stimulation was applied on the right atrial appendage to determine the effective refractory periods (ERP) and the window of vulnerability (WOV). Tumor necrosis factor α (TNF-α) and interleukin 6 (IL-6) levels in plasma and atrial tissue were measured by ELISA. Cx43, Toll-like receptor 4 (TLR4) and nuclear factor κB (NF-κB) proteins were analyzed using western blotting or immunohistochemistry. Administration of LPS for 2 weeks increased the concentration of TNF-α and IL-6 in the plasma and right atrium. ERP was markedly shortened and cumulative WOV was significantly widened in the LPS group. Following treatment with LPS, the amount of Cx43 protein in the area of intercalated disk increased. In addition, a high-density of Cx43 in the lateral connection was identified. LPS also induced the activation of NF-κB in the canine atrium. Administration with the α1-AR blocker doxazosin prevented the production of LPS-induced inflammatory cytokine and reversed the enhanced vulnerability to atrial fibrillation. Doxazosin inhibited the LPS-induced increase in Cx43 protein and heterogeneous distribution, and prevented the activation of NF-κB. These results indicated that chronic low-grade system inflammation may increase the inducibility of AF in a canine model. The underlying mechanism may be involved in the LPS-induced activation of NF-κB, and the increase in Cx43 expression and lateral distribution via an α1-AR-dependent pathway.

Introduction

Atrial fibrillation (AF) is the most common cardiac arrhythmia (1,2). The majority of patients with AF have underlying cardiovascular diseases, including valvular heart disease, coronary artery disease, cardiomyopathy and diabetes mellitus (3). A number of studies have indicated that inflammation serves an important role in the pathogenesis of AF (4-6). Patients with AF have been identified to have an elevated level of serum inflammatory markers including C-reactive protein (CRP), interleukin 6 (IL-6), tumor necrosis factor α (TNF-α) and monocyte chemoattractant protein-1 (MCP-1) (5,7,8). Histological studies have demonstrated that inflammatory cell infiltration increases in the atrial myocardium of patients with AF (6,9). Treatment with anti-inflammatory agents has been demonstrated to decrease the recurrence and perpetuation of AF (10,11). In addition, alterations in the expression of connexins and remodeling is involved in the pathogenesis of AF (12). Connexin 43 (Cx43) is one of the major connexin isoforms in the atrial myocardium (13). Previous studies have indicated that there is a link between Cx43-mediated gap-junction coupling and atrial arrhythmias (14-16). Under physiological conditions, Cx43 is localized in intercalated disks between atrial myocytes. Lateralization of Cx43 has been observed in patients with AF (14) and in the chronic pressure overload-induced AF animal model (17). However, it remains unclear whether Cx43 is involved in inflammation-induced atrial fibrillation.

There is an increasing body of evidence that demonstrates that there may be cross-talk between α-adrenergic receptor (α-AR) signaling and the immune system during inflammation. Flierl et al (18) reported that phagocytes are capable of de novo production of catecholamines when phagocytes are

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exposed to lipopolysaccharides (LPS), producing a blockade of α2-AR, which suppressed lung inflammation. A significant increase in α1-AR expression has been observed in human periodontal ligament fibroblasts following LPS pretreatment and blocking α1-AR signaling prevents the upregulation of inflammatory-associated cytokines (19). In high altitude native rats, blockage of the α-AR also induced a complete decrease in inflammatory mediators (20). Therefore, it was hypothesized that α1-adrenergic activation may be involved in inflammation-induced AF.

In the present study, dogs were administrated a low dose of LPS for 2 weeks to mimic the chronic low-grade system of inflammation. The roles of Cx43 and α1-AR in inflammation-induced AF were then investigated.

Materials and methods

Animals. A total of 20 healthy male beagles (weight, 10-12 kg; age, 12-15 months old) from the Experimental Animal Center of Hangzhou Normal University (Hangzhou, Zhejiang, China), with no prior symptoms of inflammation or AF, were used. The dogs were housed in individual cages in a controlled room (18 to 24°C with a 12/12 h light/dark cycle) for 2 weeks prior to the experiment and were given free access to food and water. The investigation conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (21). All experimental protocols were approved by the Ethics Committee on Experimental Animal Center of Hangzhou Normal University (Hangzhou, China).

Drugs and solutions. LPS (derived from Escherichia coli 055:B5) and doxazosin were purchased from Sigma-Aldrich; Merck KGaA (Darmstadt, Germany). Sodium pentobarbital was purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Cx43 (cat. no. #3512), nuclear factor κB (NF-κB) p65 (cat. no. #8242), histone H3 (cat. no. #9717) and β-actin (cat. no. #4970) antibodies were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). Toll-like receptor 4 (TLR4) antibody (cat. no. CATA00) and IL-6 (cat. no. CA6000) were purchased from R&D Systems, Inc. (Minneapolis, MN, USA).

Experimental protocol. The dogs were divided into four groups of 5 dogs each. Dogs in the control group were injected with the same volume of vehicle [0.9% NaCl, 0.2 ml/kg, intraperitoneal (i.p.)] and fed empty capsules once a day for 2 weeks. In the LPS group received LPS (0.1 μg/kg in 0.9% NaCl, i.p.) and fed empty capsules once a day for 2 weeks. In the LPS + doxazosin group, the dogs were fed α1-AR antagonist doxazosin (0.2 mg/kg) in the form of a capsule 5 min following LPS injection. Dogs in the doxazosin group were fed a capsule containing doxazosin (0.2 mg/kg) 5 min following an i.p. injection of 0.9% NaCl.

Vital signs monitoring. Vital signs (i.e., rectal temperature, heart rates and blood pressure) were observed and recorded at time 0 h (prior to the experiment) and 3, 6, 12 and 24 h following each injection of LPS. For noninvasive measurement of blood pressure, a cuff was placed on the right femoral region and connected to a commonly used noninvasive blood pressure monitor. Heart rate was measured by palpations of the femoral pulse.

Measurement of systemic TNF-α and IL-6 levels. Blood (2 ml) was collected in EDTA-coated tubes at 3 h and then 1, 4, 7 and 14 days post-treatment with the first injection of LPS. The plasma was separated by centrifugation at 2,000 x g for 10 min at room temperature, and then stored at -80°C until analysis. The systemic TNF-α and IL-6 levels were determined by Canine TNF-α Quantikine ELISA kit (cat. no. CATA00) and Canine IL-6 Quantikine ELISA kit (cat. no. CA6000) according to the manufacturer's instructions (R&D Systems, Inc.).

Animal preparation. Following 2 weeks of the different treatment, the dogs were fasted for ≥10 to 12 h and then anesthetized with sodium pentobarbital (30 mg/kg, i.p.). A multi-electrode catheter (Cordis Webster, Inc.; Biosense Webster, Inc.; Johnson & Johnson, New Brunswick, NJ, USA) was introduced from the right external jugular vein and was placed in the right atrial appendage to record right atrial electrograms and for atrial pacing. The pacing and recording leads were connected to a cardiac electrophysiology stimulator (model DF-5A; Suzhou Dongfang Electronic Instrument Factory, Shuzhou, China) and a multichannel electrophysiological recording system (model TOP-2001; Shanghai Hongtong Industrial Co., Shanghai, China). ECGs were recorded with the use of bipolar percutaneous electrodes placed in each of the dogs’ four limbs. Body temperature was maintained at 36.5±1.5°C using a heating pad situated under the dog. Anesthesia was maintained with 6 mg/kg of sodium pentobarbital i.p. administration hourly.

Programmed stimulation. Programmed stimulation was used to determine the atrial effective refractory periods (ERP) and window of vulnerability (WOV). The right atrium was paced at an atrial pacing cycle length of 300 msec; electrical pacing was repeated every 300 msec with each pace lasting 0.5 msec in duration. The ERP at 2x, 4x and 10x diastolic threshold was determined by programmed stimulation of the right atrial appendage, which consisted of 8 consecutive stimuli (S1S1=300 msec) followed by a premature stimulus (S1S2). The SIS2 intervals were decreased from 200 msec to refactoriness by decrements of 2 msec. As the SIS2 intervals approached the ERP, decrements were reduced to 1 msec. The atrial ERP was defined as the longest S1-S2 interval that failed to induce atrial depolarization (22).

The WOV was used as a quantitative measurement of AF inducibility. AF was defined as irregular atrial rates faster than 500 bpm associated with irregular atrioventricular conduction lasting longer than 5 sec. During ERP measurements, if AF was induced by decremental SIS2 stimulation, the difference between the longest and shortest S1-S2 interval (in msec) at which AF was induced was defined as the WOV. The cumulative WOV was the sum of the individual WOVs determined at 2x, 4x and 10x threshold levels in each dog (23).

The ability of the atria to develop sustained AF was also analyzed by burst pacing at 10x threshold (S1S1=100 msec and 50 msec) for 120 sec. Sustained AF was defined as a fast...
irregular rhythm that lasted for >60 sec following cessation of burst pacing (24).

Cardiac TNF-α and IL-6 content analyses. Following the measurements for AF inducibility, right atrial tissues from all 20 dogs were harvested and homogenized thoroughly on ice in a lysis buffer (50 mM Tris-HCl, 0.1 mM EDTA-2Na, 1 mM sucrose, 0.8% sodium chloride, pH 7.4). The homogenates were centrifuged at 12,000 x g for 10 min, and the supernatant was collected and stored at -80°C until analysis. The TNF-α and IL-6 levels in the supernatant were measured using the Canine TNF-α Quantikine ELISA kit (cat. no. CATA00) and Canine IL-6 Quantikine ELISA kit (cat. no. CA6000) according to the manufacturer’s instruction (R&D Systems, Inc.). Protein concentration of the supernatant was determined using a bicinchoninic acid protein assay kit (Beyotime Institute of Biotechnology, Haimen, China) according to the manufacturer’s instructions. The cardiac TNF-α and IL-6 contents were expressed as picograms per milligram of protein.

Western blot analysis. Total proteins were obtained from right atrial myocardium by homogenization in ice-cold radioimmunoprecipitation lysis solution (Cell Signaling Technology, Inc.) containing 1% Triton X-100, phosphatase, protease inhibitors and PMSF. Nuclear protein extracts were obtained using a Nuclear and Cytoplasmic Protein Extraction kit and quantified using a bicinchoninic acid protein assay kit (Beyotime Institute of Biotechnology) according to the manufacturer’s instructions. Equal amounts of protein (20 µg) from each sample were separated by 10% SDS-PAGE and transferred to a nitrocellulose membrane (EMD Millipore, Billerica, MA, USA). The membranes were blocked for 1 h with 5% bovine serum albumin (BSA) at room temperature, then incubated overnight at 4˚C with the primary antibodies, including anti-Cx43 (1:1,000), anti-TLR4 (1:500), anti-NF-κB p65 (1:1,000), anti-β-actin (1:1,000) and anti-histone H3 (1:1,000). Following washing using TBS-T (Tris-buffered saline with 0.1% Tween-20), the membrane was incubated with a horseradish peroxidase-conjugated secondary antibody (1:1,000; goat anti-rabbit IgG (cat. no. #7074) or horse anti-mouse IgG (cat. no. #7076); Cell Signaling Technology, Inc.) for 45 min at room temperature. All reactions were detected using an enhanced chemiluminescence kit (Beyotime Institute of Biotechnology) according to manufacturer’s instructions. The experiment was repeated three times. The band intensities were analyzed with Quality One software (version 4.6.2; Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Immunohistochemistry. Immunohistochemistry staining was used to determine the localization of Cx43 in the atria. Each tissue section (5-µm thick) was deparaffinized, rehydrated and blocked with 5% BSA for 1 h at room temperature. Then, the sections were incubated with the anti-Cx43 antibody (dilution, 1:500) overnight at 4°C, followed by incubation with the biotin-conjugated secondary antibody (dilution, 1:200; cat. no. A0277; Beyotime Institute of Biotechnology) for 1 h at room temperature. Nuclei were counterstained with hematoxylin staining solution (cat. no. C0107; Beyotime Institute of Biotechnology) for 5 min at room temperature. Staining was visualized using 3,3-diaminobenzidine (DAB). The sections were then observed and photographed under the BX51 microscope (Olympus Corporation, Tokyo, Japan). To quantify staining for Cx43 in the analyzed regions, integrated optical density was calculated as the product of staining area and intensity using image analysis software (Image-Pro Plus version 6.0.0.26; Media Cybernetics, Inc., Rockville, MD, USA).

Statistical analysis. Data are expressed as the mean ± standard error and were analyzed by one-way analysis of the variance (ANOVA) with Newman-Keuls’ post hoc tests, or two-way ANOVA with Bonferroni post hoc tests as required using Prism v6.0 (GraphPad Software, Inc., La Jolla, CA, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

Effect of LPS on vital signs. The dogs in the control and doxazosin only groups had no visible symptoms including, fever, lethargy, vomiting, diarrhea or increased heart rate. In the LPS only group, all dogs treated with LPS (0.1 mg/kg; n=5) developed fever and lethargy within 3 h. Signs of increased gastrointestinal motility, such as vomiting (1/5) and diarrhea (4/5), were observed within 6 h following the first LPS administration. However, these symptoms disappeared within 24 h and did not recur throughout the study. The dogs in the LPS + doxazosin group developed the same symptoms following the first LPS treatment and all symptoms disappeared within 24 h. Heart rate increased within 6 h and peaked at 12 h, then returned to normal in both the LPS and LPS + doxazosin groups. Treatment with LPS and/or doxazosin for 2 weeks did not alter body weight, the mean arterial blood pressure or heart rate. All dogs completed the study without mortality. Data are not shown.

Effect of LPS on inflammatory factor and inducibility of AF. When compared with the control group, plasma TNF-α and IL-6 levels increased at 3 h following treatment with LPS, then returned to normal levels at 24 h following treatment. However, administration of LPS for 3 to 14 days increased plasma TNF-α concentration over 6-fold. Similarly, the level of plasma IL-6 increased by ~10-fold following 3-14 days of LPS injection (P<0.01; Fig. 1). The TNF-α and IL-6 contents in the atrial tissue were also significantly increased in the LPS group when compared with the control (P<0.01; Fig. 2). The concentrations of TNF-α and IL-6 were not altered in the doxazosin only group when compared with the control group (Figs. 1 and 2). When compared with the control group, the levels of TNF-α and IL-6 were higher in the LPS + doxazosin group. However, the TNF-α content and level of IL-6 was significantly lower in the LPS + doxazosin group when compared with the LPS only group (P<0.01; Figs. 1 and 2).

Following treatment with LPS (0.1 µg/kg, daily) for 2 weeks, ERP was significantly shortened at either 2x, 4x or 10x threshold and the cumulative WOV was significantly widened in the LPS group (P<0.01 vs. control; Fig. 3). Burst pacing at 10x threshold failed to develop sustained AF in the control group, however, it did induce the occurrence of sustained AF in 3/5 dogs treated with LPS. Droxazosin alone did not influence the ERP and cumulative WOV (P>0.05; vs.
When compared with the LPS only group, doxazosin prevented the LPS-induced decrease in ERP and increase in WOV (P<0.05; doxazosin + LPS vs. LPS; Fig. 3). In addition, AF was reported in 1/5 dogs in the LPS + doxazosin group (P<0.05 vs. LPS; data not shown).

Effect of LPS on Cx43, TLR4 and NF-κB protein expression. Results from western blot analysis demonstrated that the expression of Cx43 protein significantly increased in the LPS treated group when compared with the control group (P<0.01; Fig. 4). Immunohistochemistry analysis demonstrated that Cx43 was localized to a large extent in the intercalated disk in the canine atria of the control group. Following treatment with LPS, the amount of Cx43 protein in the area of the intercalated disk increased, and a heterogeneous distribution pattern of Cx43 was identified with a high-density in lateral connection (P<0.05; Fig. 5). Doxazosin only treatment did not influence the expression and distribution of Cx43 (P>0.05 vs. control; Figs. 4 and 5).

However, in the LPS + doxazosin group, doxazosin inhibited the LPS-induced increase in Cx43 protein and the heterogeneous distribution, when compared with LPS only (P<0.01; Figs. 4 and 5).

TLR4 protein expression in the canine atrium did not increase following 2 weeks of LPS only, LPS + doxazosin or doxazosin only administration. Although LPS treatment did not alter the amount of total NF-κB protein expression, it enhanced the nuclear NF-κB level in the canine atrial myocardium (P<0.01; Fig. 4), suggesting that LPS may induce the activation of NF-κB and promote the nuclear translocation of NF-κB in the canine atrium. Administration with doxazosin only did not alter the total and nuclear levels of NF-κB when compared with control; Fig. 3). When compared with the LPS only group, doxazosin prevented the LPS-induced decrease in ERP and increase in WOV (P<0.05; doxazosin + LPS vs. LPS; Fig. 3). In addition, AF was reported in 1/5 dogs in the LPS + doxazosin group (P<0.05 vs. LPS; data not shown).

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However, the level of nuclear NF-κB in the LPS + doxazosin group was significantly lower when compared with the LPS only group (P<0.01; Figs. 4 and 5).

Discussion

AF has traditionally been regarded as a sporadic and acquired disease, however, a large population-based cohort study and animal experimental data have suggested that local and systemic inflammationserve an important role in the initiation and perpetuation of AF (25,26). In 2007, Boos et al (27) reported that an LPS challenge as an intravenous bolus of low dose induced a significant increase in acute inflammatory indexes, however, it did not lead to the development of acute new-onset AF in a large cohort of healthy LPS-challenged subjects. However, it has been previously determined that increased risk of AF is mainly associated with low-grade chronic inflammation (28). Low-grade inflammation is induced by a number of systemic diseases including obesity, hypertension and coronary artery disease (4,26,29). Transgenic mice overexpressing TNF-α in the heart have been demonstrated to develop atrial arrhythmias (30). Perfusion with IL-6 >20 min induced an increase in the duration of the action potential in isolated rat atrial tissue and led to the appearance of atrial fibrillation (31). However, whether the subacute treatment of LPS could directly affect cardiac rhythms or induce atrial fibrillation is unclear. In the present study, a chronic low-grade system inflammation model was established by administration of 0.1 µg/kg of LPS once a day for 2 weeks. This dosage of LPS (from Escherichia coli 055:B5) can cause detectable inflammation although no severe clinical symptoms or death (32,33); however, a previous study has demonstrated that using LPS from Escherichia coli 0111:B4 with the same dosage induces severe fever, vomiting, diarrhea and even death in dogs (34). A low-grade subacute or chronic system inflammation model could be established by continuous subcutaneous infusion or repeated daily injection of low dose LPS for ~5 to 28 days (35,36). Therefore in the present study, LPS from Escherichia coli 055:B5 was used, which was induced only transient fever, vomiting and diarrhea. Although repeated administration of LPS developed an adaptive response in a set of behaviors (such as the febrile response), which was similar to the results of previous studies (36,37), it did induce a system inflammatory response and increase in the inducibility of AF.

In the present study, total Cx43 protein expression increased in the LPS group dogs, and treatment with LPS increased the expression of the Cx43 protein in the intercalated disk and also produced a high density distribution of Cx43 at the lateral
borders of atrial myocytes. Cx43 serves a crucial role in the normal function of the cardiovascular system, and acts as a crucial factor to generate arrhythmias. An increase in Cx43 was identified in patients with lone AF or AF in mitral valve repair when compared with patients in sinus rhythm (38). It has also been reported that atrial Cx43 expression increased in the canine model of pacing-induced sustained atrial fibrillation (39, 40). Somatic genetic defects of Cx43 have been reported as a potential cause of AF in patients with sporadic, nonfamilial lone AF (41). Abnormal distribution of connexins seems to serve a crucial role in the initiation and perpetuation of AF (42). Fastened side-to-side conduction velocity due to an increase in the lateral side of the gap junction may predispose the atrium to reentry (43).

The molecular mechanism of altered Cx43 protein expression and distribution during low-grade systemic inflammation-induced AF is unclear. TNF-α has been suggested as a main mediator of the inflammatory response, contributing to the development of a number of cardiovascular diseases such
as AF (30, 44). TNF-α may change the intracellular distribution of Cx43 in mouse cardiomyocytes (45, 46). The activation of the TLR4 receptor has been known as a classical pathway to increase the production of proinflammatory cytokines (such as TNF-α) through the phosphorylation of inhibitor of κB and activation of NF-κB (47). TLR4 expression has been detected in the atrium (48). In the present study, administration of low-dose LPS for 2 weeks led to the activation of NF-κB and significantly increased the levels of TNF-α and IL-6 in the atria, however, it did not alter TLR4 expression.

An increasing body of evidence has demonstrated that there is a cross-talk between α1-AR signaling and cytokine production. Catecholamines have been reported to be produced by phagocytes and enhance acute inflammatory injuries (18). In human monocytes and macrophages, α1-AR positively regulates the LPS-induced cytokine production (IL-1β) production (49). Stimulation of α2-AR augments the production of TNF-α in vitro from alveolar macrophage (50). Panama et al. (51) identified that activation of α1-AR regulates the fast transient outward K+ current in rat ventricular myocytes via the NF-κB-dependent signaling pathway. Neuronally induced atrial arrhythmias can be significantly increased the inducibility of AF in the canine model. The present study was supported by the National Natural Science Foundation of China (grant nos. 81170167, 81,270,002 and 81471837) and the Science and Technology Department of Zhejiang Province (grant no. 2015C37129).

References


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