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Prostaglandin $F_{2\alpha}$ modulates atrial chronotropic hyporesponsiveness to cholinergic stimulation in endotoxemic rats

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ABSTRACT

Endotoxemia induces various physiological adaptive responses such as tachycardia. There is evidence to show that inflammatory tachycardia might be linked to a direct action of prostanoids on the cardiac pacemaker cells. Recent reports have indicated that systemic inflammation may uncouple of cardiac pacemaker from cholinergic neural control in experimental animals; however, the exact mechanism of this phenomenon is uncertain. This study was aimed to explore the hypothesis that prostanoids modulate atrial chronotropic hyporesponsiveness to cholinergic stimulation in endotoxemic rats. Male albino rats were given intraperitoneal injection of either saline or lipopolysaccharide (LPS, 1 mg/kg). 3 h after saline or LPS injection, the atria were isolated and chronotropic responsiveness to cholinergic stimulation was evaluated in an organ bath. The expression of atrial cyclooxygenases (COX)-1, COX-2 and COX-3 mRNA was assessed by quantitative real-time RT-PCR and cytosocalcium-dependent phospholipase A_2 ($cPLA_2$) activity was measured in the atria. The expression of atrial COX-2 mRNA and $cPLA_2$ activity increased significantly in endotoxemic atria ($P<0.05$). Incubation with prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$, 100 pM) could significantly decrease chronotropic response to cholinergic stimulation *in vitro*. Likewise, LPS injection could induce a significant hyporesponsiveness to cholinergic stimulation, and incubation of isolated atria with either indomethacin (5 μM) or AL-8810 (a $PGF_{2\alpha}$ antagonist, 10 μM) could reverse it ($P<0.01$, $P<0.05$, respectively), while SQ29548 (a thromboxane A_2 antagonist, 10 nM) was failed ($P>0.05$). Our data showed that $PGF_{2\alpha}$ may contribute to the atrial chronotropic hyporesponsiveness to cholinergic stimulation in endotoxemic rats.

1. Introduction

Systemic inflammation due to bacterial infections is still a major cause of mortality in intensive care units. Among bacteria, the Gram-negative bacteria induce sepsis, which is attributed to the presence of endotoxins in the cell wall of these microorganisms that contains lipopolysaccharide (LPS). LPS has several receptors, which Toll-like 4 (TLR4) is the most important one. Stimulation of this receptor leads to activation of signaling pathways and transcription factors and results to the synthesis of prostanoids and cytokines (Adham et al., 2012; Fan et al., 2013; Takayama et al., 2005). There is continuous expression of TLR4 in heart, particularly in atrial and ventricular myocytes (Grinko et al., 1995; Jazaeri et al., 2013).

Endotoxemia induces various physiological adaptive responses such as tachycardia. Although tachycardia is one of the cardinal manifestations of endotoxemia, the mechanism of endotoxin-induced tachycardia is not well-understood. It was initially thought that inflammation-associated tachycardia is linked to increased sympathetic activation. However, there is evidence against this hypothesis as beta-blockers do not block the effect of systemic inflammation on heart rate in mice (Takayama et al., 2005). Takayama et al. (2005) have shown that inflammatory tachycardia might be due to a direct action of prostanoids on the cardiac pacemaker cells. Recent reports have also indicated that systemic inflammation may uncouple the cardiac pacemaker cells form cholinergic neural control in experimental animals; however, the exact mechanism of this phenomenon is not well-understood (Gholami et al., 2012). This study was aimed to explore the

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hypothesis that prostanoids modulate atrial chronotropic hyporesponsiveness to cholinergic stimulation in endotoxemic rats.

2. Materials and methods

2.1. Chemicals

All reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless remarked otherwise.

2.2. Animals

Male Wistar albino rats (weighting 230–250 g) were provided from the Department of Pharmacology (Tehran University of Medical Sciences). Animals were given *ad libitum* access to standard rodent chow and water, with a light/dark cycle of 12 h, at a temperature of 22 ± 2 °C and 80% humidity. All animal procedures were in accordance with Guide for the Care and Use of Laboratory Animals (NIH US publication, no. 85-23, revised 1985) recommendations.

2.3. Experimental plan

For the chronotropic response of isolated atria, the animals were divided into 12 groups, randomly. Eight groups received 0.5 ml of normal saline, while four groups were injected by endotoxin (lipopolysaccharide [LPS]; *Salmonella typhimurium* LPS, 1 mg/kg dissolved in isotonic saline) intraperitoneally, 3 h before the isolation of atria. Saline-treated groups were subdivided to eight groups: vehicle (ethanol 0.0005%), indomethacin (5 μM), prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$, 100 pM), AL-8810 ($PGF_{2\alpha}$ antagonist, 10 μM), I-BOP (thromboxane A_2 analog, 10 pM, Cayman chemical, Michigan, USA), SQ29548 (thromboxane A_2 antagonist, 10 nM, Cayman chemical, Michigan, USA), AL-8810 30 min prior to adding $PGF_{2\alpha}$, and SQ29548 30 min before incubation of thromboxane A_2 (TXA_2) into organ bath. LPS-treated groups were subdivided to four groups: vehicle (ethanol 0.0005%), indomethacin (5 μM), AL-8810 (10 μM), and SQ29548 (10 nM) in organ bath. 30 min after drug incubation, the responsiveness of isolated atria to carbacholine (10^{-8} to 10^{-5} M) was assessed in all groups. Six to eight animals were used in each experimental group. For real-time reverse transcription-polymerase chain reaction (RT-PCR) study, six rats were used in either of saline- and LPS-treated groups (12 animals in total) and finally four rats were used for measuring the cytosolic calcium-dependent phospholipase A_2 ($cPLA_2$) activity in both of saline- and LPS-treated groups (eight animals totally).

2.4. Preparation of isolated atria

After induction of anesthesia with ketamine (80 mg/kg, Alfasan, The Netherlands) and diazepam (2 mg/kg, Caspian Tamin, Iran) intraperitoneally, the heart was quickly removed. The atria were carefully dissected from the ventricles, attached to a tissue holder and immersed into an organ bath containing 20 ml of carbogenated (95% oxygen and 5% CO_2) physiologic slat solution at 37 °C and pH 7.4. The composition of the solution was as follows (mM): NaCl 112.0, KCl 5, $CaCl_2$ 1.8, $MgCl_2$ 1.0, NaH_2PO_4 0.5, KH_2PO_4 0.5, $NaHCO_3$ 25.0, glucose 10.0, and EDTA 0.004 (Merck, Germany). A preload tension of 1000 mg was applied to the atria and tissues were allowed to equilibrate for 30 min (Mani et al., 2006a). The responsiveness of isolated atria to cholinergic stimulation was then evaluated by addition of cumulative concentrations of carbacholine (10^{-8} to 10^{-5} M) to the organ bath. The rate of spontaneous contractions was recorded by the isometric force transducer of a PowerLab system and atrial beats were calculated using the LabChart software (ADInstrument, Australia). IC_{50} values were calculated using nonlinear regression analysis (GraphPad Prism 5 software, San Diego, CA, USA).

2.5. mRNA extraction and real-time reverse transcription-polymerase chain reaction (RT-PCR)

RT-PCR was used to study the expression of cyclooxygenase (COX)-1, COX-2, COX-3, prostaglandin $F_{2\alpha}$ receptor (FP) and thromboxane A_2 receptor (TP) in isolated atria. The rat atria were isolated and immediately immersed in liquid nitrogen. Total RNA was extracted from tissue homogenate using RNeasy Fibrous Tissue mini kit (Qiagen, Germany). The first strand cDNA was then made by using 1 μg of deoxyribonuclease treated RNA, 1 μl of random hexamer primer (p(dN)6), and ribonuclease free water, heated at 70 °C for 5 min, and then placed on ice. RNasin (ribonuclease inhibitor), 100 unit of Moloney murine leukemia virus (MMLV) reverse transcriptase, MMLV buffer, and 0.4 mM deoxynucleoside triphosphates were added, and the mix was incubated at 42 °C for 1 h. The oligonucleotide primers used for PCR amplification were as follows:

Rat COX-1, Forward: 5′-TCCTACATGGGATGACGAGC-3′, Reverse: 5′-GGTTGCGATACTGGAACTGG-3′

Rat COX-2, Forward: 5′-CCCTGAAGCCGTACACATCA-3′, Reverse: 5′-TGTCACTGTAGAGGGCTTTCAATT-3′

Rat COX-3, Forward: 5′-AGTCATGAGTCGTGAGTCCG-3′, Reverse: 5′-ACTGGAGCGAGAGACTCCTT-3′

Rat FP, Forward: 5′-ACAGGCAAGGCAGGTCTC-3′, Reverse: 5′-AGCGTCGTCTCACAGGTC-3′

Rat TP, Forward: 5′-GTGAGGTGGAGATGATGGTTC-3′, Reverse: 5′-CGTAGGTAGATGAGCAGTTGG-3′

Rat 18S, Forward: 5′-ATCACCTTTCGATGGTAGTCG-3′, Reverse: 5′-TCCTTGATGTGGTAG CC-3′

Real-time PCR reaction was included of 2 μl of cDNA template, 10 pmol each of forward and reverse primers, 6 μl distilled water, and 10 μl of optimized PCR mastermix (Promega, UK) in a total reaction volume of 20 μl, which performed with a Rotor-Gene machine (Qiagen, Germany). We used 18S rRNA as an internal control gene for normalization of real-time PCR data. Thermal cycling protocol was as follows: initial denaturation step (15 min at 95 °C), followed by 40 cycles of denaturation step (45 s at 95 °C), annealing step (45 s at 57.1 °C for TP and 18S, 58 °C for COX-2, and 65 °C for COX-1, COX-3 and FP) and elongation step (45 s at 72 °C). The level of transcriptional difference between endotoxin-treated group and control group was calculated relative to the level of 18S RNA expression.

2.6. Measurement of $cPLA_2$ activity

Phospholipase A_2 activity was measured by a $cPLA_2$ Assay Kit (Cayman chemical, Michigan, USA). Briefly, each atria was homogenized in 1 ml of phosphate buffered saline (PBS, pH 7.4), then centrifuged at 10,000*g* for 15 min at 4 °C. $cPLA_2$ activity was measured by adding 10 μl of sample supernatant to plate wells. To eliminate measurement of any calcium independent PLA_2 activity in the samples, bromoenol lactone, a specific inhibitor of $iPLA_2$ (5 μM) dissolved in dimethylsulfoxide (DMSO) was added and incubated at 25 °C for 15 min. The reaction was started by the addition of 200 μl of the substrate, arachidonoyl-thio-PC (1.5 mM), shaking the plate for 30 s, and incubating for 60 min at room temperature. A positive control of bee venom PLA_2 (10 μl) was diluted in 5 μl DMSO and used in the assay. Then, by addition of 10 μl of DTNB/EGTA to each well, the enzyme activity stopped. The plate was carefully shaken, and incubated for 5 min at room temperature. Absorbance was read at 414 nm using a plate reader (Synergy HT, Biotek, USA). The reaction rate (absorbance 414/min) was determined by subtraction of the absorbance of the