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A potential relationship between gut microbes and atrial fibrillation: Trimethylamine N-oxide, a gut microbe-derived metabolite, facilitates the progression of atrial fibrillation

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ABSTRACT

Background: Emerging evidence indicates gut microbes and their products could activate the autonomic nervous system (ANS), which plays important roles in the initiation and maintenance of atrial fibrillation (AF). Trimethylamine N-oxide (TMAO), a metabolite derived from gut microbes, is associated with cardiovascular diseases. The present study aimed to investigate the role of TMAO in the progression of AF.
Methods: In part 1: TMAO or saline was locally injected into 4 major atrial ganglionated plexi (GP) to clarify its effect on cardiac ANS and AF inducibility in normal canines. In part 2: TMAO or saline was injected into 4 major atrial GP to test its effect on AF progression in a rapid atrial pacing (RAP)-induced AF model.
Results: In part 1: Local injection of TMAO significantly increased anterior right GP (ARGP) function and neural activity, shortened ERP values. In part 2, compared with the control group, 6-hour RAP significantly shortened the ERP, widened the $\sum$WOV, enhanced the ARGP function and neural activity, increased the NGF and c-fos expression, and up-regulated the inflammatory cytokines. TMAO aggravated all of these changes by activating the proinflammatory p65 NF-κB signaling pathway.
Conclusions: TMAO could increase the instability of atrial electrophysiology in normal canines and aggravate the acute electrical remodeling in a RAP-induced AF model by exacerbating autonomic remodeling. The increased inflammatory cytokines in the GP due to the activation of p65 NF-κB signaling may contribute to these effects.

1. Introduction

A diverse microbial community consisting of 1000 to 1500 bacterial species colonizes the human gut, and the number of genes associated with gut microbes might exceed those of the host by approximately 150 times [1,2]. Commensal gut microbes are important components of the digestive system and contribute to host metabolism. Beyond the role they play in shaping gut metabolism, these microbes also affect non-gut organs (such as the brain and heart) and metabolic diseases [3–5].

Accumulating evidence links gut microbes and cardiovascular diseases [4,6]. Trimethylamine N-oxide (TMAO) is an important gut microbe-derived metabolite linked to cardiovascular diseases [7]. Using a metabonomics approach, TMAO was identified clinically as a predictor of cardiovascular events [4]. Other studies indicated that TMAO promotes atherosclerosis and induces platelet hyperreactivity [8,9]. Moreover, gut microbes and their products are important contributors to the development of nervous system and can activate the autonomic nervous system (ANS) [10–12]. It is known that the cardiac ANS (CANS) plays an important role in the initiation and maintenance of atrial fibrillation (AF) [13,14]. The ganglionated plexi (GP) located in the epicardial fat pads integrate the neural trafficking in the CANS. Both clinical and preclinical studies showed that stimulation of the intrinsic CANS, including the atrial GP, promotes AF [15,16], whereas the suppression of these GP prevents AF [14,17].

The concept of "AF begets AF" denotes that AF is a self-perpetuating arrhythmia and AF itself is the most important predictor of its own progression [18,19], but how AF begets itself in the first few hours after its initiation remains poorly understood. Previous studies have shown that the CANS plays a critical role in the process of "AF begets AF" [14,20].

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Autonomic remodeling and atrial electrical remodeling form a vicious cycle to perpetuate AF in the first few hours [20]. AF is also known to be highly associated with inflammation [21]. In the present study, we hypothesized that TMAO may facilitate the progression of AF by activating the CANS and pro-inflammatory pathways.

2. Methods

2.1. Animal preparation

The 21 mongrel dogs weighing 20 to 24 kg were used in this experiment, and all the animals were supplied by the Animal Center at Renmin Hospital, Wuhan University. The Animal Ethics Committee of Wuhan University approved all of the study protocols, which complied with the "Guide for the Care and Use of Laboratory Animals" published by the US National Institutes of Health.

All surgeries were performed under anesthesia with 3% pentobarbital sodium, and the animals were ventilated with a positive pressure respirator with room air. A bilateral thoracotomy was performed to expose the dogs' bilateral atriums, atrial appendages, pulmonary veins (PVs), and atrial GPs. An intravenous infusion of saline was supplied to compensate for fluid loss during the procedure. A computer-based laboratory system (Lead 7000, Jinjiang Inc., Chengdu, China) was used to record surface electrocardiograms throughout the procedure. The animals were euthanized with an overdose intravenous injection of pentobarbital sodium (100 mg/kg) at the end of the experiment.

2.2. Study protocol

In part 1, local injection of TMAO (Sigma, St. Louis, Mo., USA) or saline into 4 major atrial GP (the anterior right GP (ARGP), the inferior right GP, the superior left GP, and the inferior left GP) was applied to test the effect of TMAO on CANS and AF inducibility in normal canines.

In part 2, TMAO was locally injected into 4 major atrial GP to determine its' direct effect on CANS and the progress of AF in a rapid atrial pacing (RAP) induced AF model. The animals were randomly divided into control group ($n = 6$, saline injection into 4 major without RAP), RAP group ($n = 6$, saline injection into 4 major GP plus 6 h of RAP), and TMAO + RAP group ($n = 9$, TMAO injection into 4 major GP plus 6 h of RAP). ARGP function and neural activity, as well as ERP values were determined at different time points. The protocol is outlined in Supplemental Fig. S1.

2.3. TMAO injection

A bilateral thoracotomy was performed to expose 4 major atrial GP: the ARGP, the inferior right GP, the superior left GP, and the inferior left GP. To demonstrate TMAO could directly activate atrial GP and avoid its indirect influence (gut-brain-heart axis, Fig. 4A) on atrial electrophysiological properties, 300 nmol TMAO (0.3 mL 1 mmol/L, dissolved in saline) or equivalent saline was very locally injected into the epicardial fat pads containing 4 major atrial GP rather than the blood vessel before RAP. The TMAO dose used in the present study was based on the dosage reported by the team discovering the relationship between TMAO and cardiovascular disease [22]. In their previous studies, acute intraperitoneal injection of 86 μmol TMAO was performed in mice and produced a peak circulating TMAO level to about 100 μmol/L, which was similar to the levels in mice chronically fed with choline-supplemented diet and in some clinical reports. According to the equivalent dose conversion between species based on body surface area [23], the dosage of TMAO in the present study was supposed to be about 11,300 μmol, but a much lower dosage (300 nmol to each GP) was used in the present study because of the very local application of TMAO. Each injection was slowly performed for approximately 1 min.

2.4. RAP and the measurement of atrial electrophysiological properties

RAP (20 Hz, 2 × threshold) was performed at the left atrial appendage to induce acute atrial remodeling. After 3 h of RAP, pacing was stopped temporarily for approximately 10 min to measure the atrial electrophysiological properties (see below). These electrophysiological properties were measured again at the end of 6 h of RAP. Programmed stimulation was delivered to all atria, atrial appendages and PVs sites to determine the effective refractory period (ERP) and window of vulnerability (WOV) as previously described [20]. The WOV was defined as the width between the longest and shortest S1S2 coupling intervals at which AF was induced. The WOVs at all sites were calculated as the $\sum$WOV, which represents a measurement of AF inducibility as previously reported [20,24].

2.5. Measurements of GP function and neural activity

Multi-electrode catheters were attached to the ARGP. The function of ARGP was measured as the sinus rate slowing responses to high frequency stimulation (HFS, 20 Hz, 0.1 ms pulse width) as previously described [20,24]. Because the sinus rate slowing responses of each dog to HFS showed significant variation, delivering the same voltages for HFS was impossible. We therefore used four incremental voltage levels to stimulate the ARGP (level I:1–4 V; level 2: 5–7.5 V; level 3: 7.5–10 V; level 4: 10–15 V). Data were presented as the percent of maximal sinus rate slowing (compared to the baseline state) induced by ARGP stimulation at different levels of stimulation voltages (Fig. 2B). Two tungsten micro-electrodes were inserted into the fat pad containing the ARGP, and a PowerLab data acquisition system (8/35, AD Instruments, New South Wales, Australia) was used to record and analyze the signals generated from the ARGP at different time points. Signals with three times higher amplitudes than the baseline noise were identified as the neural activity within ARGP. The amplitude and frequency of the signals were analyzed to quantitatively characterize the ARGP activities.

2.6. Quantitative real-time polymerase chain reaction (RT-PCR)

The ARGP tissues were stored at −80 °C. After homogenization, RNA was extracted from the ARGP tissues using TRIzol reagent (Invitrogen Life Technologies) according to the manufacturer's protocol. Total RNA was reverse transcribed with a RevertAid First Strand cDNA Synthesis Kit (Thermo). The expression levels of the candidate genes were measured using RT-PCR with FastStart Universal SYBR Green Master Mix (Roche) and quantified via a RT-PCR system (Applied Biosystems 7300). The mRNA levels of each target gene were calculated using the relative standard curve method and normalized to the GAPDH mRNA levels from the same samples. All RT-PCR primer sequences listed in Supplemental Table S1 were obtained from Invitrogen Biotechnology Co., LTD.

2.7. Western blot analysis

Fresh ARGP tissues were immediately excised at the end of the experiment and stored at −80 °C. After tissue homogenization in buffer on ice, supernatants were collected as total lysate. Equal amounts of homogenate proteins were loaded onto a 4–20% Gradient Minigel (CPL, Austin, Texas) and then electrically transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore). The membranes were blocked with 5% nonfat dry milk and then incubated with primary antibodies (IL-1β, Abcam; IL-6, PTG; TNF-α, Abcam; nerve growth factor (NGF), Abcam; c-fos, Abcam; p65 nuclear factor kappa B (NF-κB), CST; p-p65-NFκB, Bioworld) at 4 °C overnight, washed fully in TBST, and incubated with anti-rabbit secondary antibody. Then, the antibody-binding protein bands were visualized and quantified.

2.8. Histological studies

The ARGP tissues were excised quickly at the end of the experiment and fixed in 4% paraformaldehyde at room temperature. Paraffin blocks were processed and cut into 5-μm sections. Immunofluorescent antibodies (NGF, Abcam; c-fos, Abcam) were used to detect the expression of NGF and c-fos, and a quantitative analysis was conducted using commercially available software (Image Pro Plus, Media Cybernetics, Inc., Rockville, MD).

2.9. Statistical analysis

All continuous data are presented as the mean ± SEM. A one-way analysis of variance followed by a Bonferroni post hoc test was used for multiple comparisons with regard to the IL-1β, IL-6, TNF-α, c-fos, and NGF levels among the different groups. A two-way repeated-measures analysis of variance was used to analyze the differences between the ERP, $\sum$WOV, and ARGP function among different groups at different time points. Prism software (version 6.0, GraphPad Software, Inc., San Diego, California) was used to analyze these data and create graphs. Values were considered as significant at $P < 0.05$.

3. Results

3.1. Part 1: the effect of TMAO on CANS and AF inducibility in normal canines

To test whether TMAO alone could activate the CANS and increase AF inducibility, TMAO was locally injected into 4 major atrial GP in normal canines. In comparison with the control group, microinjection of TMAO significantly increased the ARGP function (Supplemental Fig. S2), as well as the firing frequency and amplitude of neurons within ARGP (Supplemental Fig. S3). Meanwhile, TMAO significantly shortened the ERP values at all sites (Supplemental Fig. S4). These results indicate that TMAO alone could activate the CANS and increase AF inducibility in normal canines.

3.2. Part 2: the effect of TMAO on CANS and AF progression in an RAP-induced AF model

3.2.1. Effect of TMAO on ERP and $\sum$WOV

Compared with the control group, RAP significantly shortened the ERPs at the 3- and 6-hour time points, whereas RAP + TMAO further shortened the ERPs compared to RAP alone. For instance, at the LSPV site, no significant differences were observed at baseline. At the 3-hour time point, the ERP values were 129.3 ± 2.4 ms, 113.7 ± 2.6 ms, and 95.0 ± 4.7 ms in the control group, RAP group and TMAO + RAP