Eicosapentaenoic acid prevents atrial fibrillation associated with heart failure in a rabbit model

Kazuhisa Kitamura, Rei Shibata, Yukimi Tsuji, Masayuki Shimano, Yasuya Inden, and Toyoaki Murohara

1Department of Cardiology, Nagoya University Graduate School of Medicine, 2Department of Cardiovascular Research, Research Institute of Environmental Medicine (RIEM), Nagoya University, Nagoya, Japan

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ATRIAL FIBRILLATION (AF) is common in patients with heart failure (HF) and is associated with increased morbidity and risk of mortality in patients with HF (41). AF and its duration are associated with atrial fibrosis, which causes conduction abnormalities through the atria and creates a substrate for AF by itself particularly in the state of HF (3, 16). Antiarrhythmic drug therapies to prevent AF in patients with HF are limited by their toxicity and intolerance (42). Therefore, it is clinically valuable to discover alternative therapies for HF-related AF.

Dietary consumption of fish oil is associated with a reduced incidence of cardiovascular events. Recent epidemiological and experimental studies reported that high consumption of fish oil may prevent the development and progression of HF (37). With regard to AF, prospective cohort studies found an inverse association of AF with intake of fish oil (20). High serum concentration of total long-chain n-3 polyunsaturated fatty acids (PUFAs) was associated with a reduced risk of AF in men (40). In contrast, two prospective studies have shown that higher intake of fish oil was not associated with reduced onset of AF (4, 9). Thus, in epidemiological studies, the relationship between the amount of intake of fish oil and the prevention of AF is still controversial.

Active components in fish oil are considered to be n-3 PUFAs, especially eicosapentaenoic acid (EPA, C20:5n-3), and docosahexaenoic acid (C22:6n-3) (2). It was shown that consumption of highly purified EPA, one class of PUFAs used clinically to treat hyperlipidemia, reduced the risk of major cardiovascular events (7, 11, 43). Recent experimental reports show that PUFAs may prevent AF and atrial vulnerability to arrhythmia in several animal models (15, 26, 27). However, it is still unknown how EPA exhibits beneficial actions against AF. Here, we investigated the effect of EPA on AF associated with HF in a novel rabbit tachypacing model.

MATERIALS AND METHODS

Antibodies to tumor necrosis factor-α (TNF-α, sc-1348; goat), AMP-activated protein kinase (AMPK)-α1 (sc-19128; goat), phosphorylated AMPK-α1 (sc-33524; rabbit), and phosphorylated extracellular signal-regulated kinase (ERK, sc-61982; goat) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA); tumor necrosis factor-α (TNF-α, sc-1348; goat), adiponectin (MAB1119; mouse) was purchased from R&D Systems (Minneapolis, MN); and GAPDH (ab8245; mouse) was purchased from Abcam (Cambridge, UK).

Rabbit model of ventricular tachypacing and experimental protocol. Male New Zealand white rabbits (2.5 to 3.5 kg), at the age of 14 to 25 wk, were used (Kitayama Labs, Kyoto, Japan). The study protocol was approved by the Institutional Animal Care and Use Committee of Nagoya University School of Medicine. Programmable right ventricular pacemakers (Medtronic, Minneapolis, MN) were implanted as described previously (39, 31). The pacemakers were programmed to pace at 380 beat/min for 28 days. Echocardiographic analysis and open chest electrophysiological studies were performed at 4 wk after surgery. Rabbits were divided into three groups as follows: nonpaced control rabbits, rabbits subjected to ventricular tachypacing without treatment of EPA (VTP rabbits), and rabbits subjected to VTP with treatment of EPA (VTP + EPA rabbits).

Echocardiography. To measure chamber dimensions and cardiac function, echocardiography was performed with Apio SSA-700A machine using a 15-MHz probe (Toshiba, Tochigi, Japan) before and at 28 days after pacemaker implantation. Two operators performed the measurements at random. After a good-quality two-dimensional in-
age was obtained. M-mode images of the left ventricular dimension, wall thickness, and fractional shortening were measured.

**Electrophysiological study.** To exclude acute antiarrhythmic effects, administration of EPA was discontinued at 48 h before electrophysiological study. At day 28 after pacemaker implantation, rabbits were anesthetized with ketamine hydrochloride (35 mg/kg im) and xylazine (3 mg/kg im), after which anesthesia was maintained using isoflurane (0.5–3.0%). Rabbits were ventilated with a positive-pressure respirator, after which the pacemaker was deactivated. Medial sternotomy was performed, and custom-made bipolar electrodes were hooked into the right and left atrial appendages (RAA and LAA) for recording and stimulation. A programmable stimulator (Nihon, Koden, Japan) was used to deliver twice-threshold currents at a 2-ms pulse duration. Effective refractory periods (ERPs) were measured at RAA and LAA at basic cycle lengths of 300, 200, and 150 ms with a train of 20 basic stimuli (S1) followed by a premature extrastimulus (S2) at 2-ms decrements. The ERP was defined as the longest S1-S2 diastolic threshold current. AF was defined as a rapid (≤500/min) AF inducibility was defined as the time from the RAA pacing spike to LAA activation per interval failing to capture the atria. The conduction velocity was defined as the rate of first irregular rhythm lasting more than 0.5 s after cessation of burst pacing.

**Histology.** Atrial tissues were obtained at 28 days after pacemaker implantation. Tissue samples were embedded in optimal cutting temperature compound (Miles, Elkhart, IN) and snap-frozen in liquid nitrogen. Five-micron tissue slices were prepared and stained with Masson trichrome for evaluation of the extent of fibrosis. To quantify the percent fibrosis area of the LA free wall, the blue pixel content of digitized images was measured relative to total tissue area using a thresholding function of the analysis program ImageJ (NIH, Bethesda, MD). Blood vessels and perivascular interstitial tissues were excluded from the fibrosis quantification. The averaged number of transverse sections of the LA free wall for quantitative analysis was 30 for each rabbit.

**Western blotting.** Atrial and epicardial adipose tissue samples obtained at 7 and 28 days after pacemaker implantation were homogenized in lysis buffer containing 20 mM Tris-HCl (pH 8.0), 1% NP-40, 150 mM NaCl, 0.5% deoxycholic acid, 1 mM sodium orthovanadate, and protease inhibitor cocktail (Sigma Chemical, St. Louis, MO). Identical amounts of protein were separated with denaturing SDS 10% polyacrylamide gels. The membranes were immunoblotted with the primary antibodies at a 1:1,000 dilution followed by secondary antibody at a 1:5,000 dilution. Bands were visualized using ECL Western Blotting Detection Kit (Amersham Pharmacia Biotech, Piscataway, NJ).

**Real-time RT-PCR.** Total RNA from atrial and epicardial adipose tissues obtained at 7 and 28 days after pacemaker implantation was isolated with the use of guanidium isothiocyanate phenol chloroform solution (TRIZol reagent; Invitrogen Life Technologies, Tokyo, Japan). The cDNA was produced using oligo-dT primer and superscript II reverse transcriptase (superscript II, Invitrogen Life Technologies). Real-time RT-PCR was performed with 1-µg cDNA on an Mx3000P Real-Time PCR System (Stratagene/Agilent Technologies, La Jolla, CA) using SYBR Green I as a double-stranded DNA-specific dye in accordance with the manufacturer's instructions (Life Technologies). Primers were as follows: 5'-CGGAGCTGATGGTGATCCGCG-3' and 5'-GGCATGAACTACCCGCACAC-3' for TNF-α; 5'-CGGACGCTTACAGCTCATG-3' and 5'-GCCACGCTATCGTGACG-3' for TGF-β; 5'-CTCTTGGGTCTGTGGCATTC-3' and 5'-GAAATCCTGTTTGCACCTTTATG-3' for collagen type I; 5'-CACACCTTCTCTGAACGT-3' and 5'-ATTATGACCGACTTGAGAC-3' for collagen type III; and 5'-CGGAGCCAAAAAGGTGCTACAT-3' and 5'-TTTCCAGCCGCCAGTGTCAG-3' for GAPDH genes.

**Effect of EPA on VTP-induced HF.** There were no significant differences in body weight and blood pressure among the experimental groups. Table 1 summarizes echocardiographic parameters in VTP rabbits and VTP + EPA rabbits at baseline and at 28 days after pacemaker implantation. Left atrial diameter (LAD), LV end-systolic diameter (LVESd), and LV end-diastolic diameter (LVEDd) were significantly increased in response to VTP in rabbits at 28 days. VTP for 28 days also decreased fractional shortening (FS). Treatment with EPA did not affect the VTP-induced increase in LAD, LVESd, and LVEDd and the decreased FS (Table 1). There were no significant differences in mortality between VTP and VTP + EPA rabbits (45% in VTP rabbits and 41% in VTP + EPA rabbits). Thus continuous VTP for 28 days induced HF status in these rabbits, but the EPA treatment did not affect VTP-induced HF conditions.

Effect of EPA on AF. ERPs at the RAA and LAA are shown in Fig. 1, A and B. VTP tended to increase ERPs, but there were no significant differences among the groups. Figure 1C shows conduction velocity (CV) between the LAA and RAA. CV was significantly decreased by 45% with VTP (P < 0.005), whereas treatment with EPA did not affect CV in response to VTP.

Figure 2A shows representative images of AF induced by burst pacing at the RAA and LAA from each group. Quantitative analysis revealed the VTP resulting in an increased DAF following burst pacing, whereas little or no AF was induced in rabbits without VTP. EPA treatment significantly attenuated the increase in DAF of VTP rabbits (DAF from RA; VTP rabbits without VTP. EPA treatment significantly attenuated the increase in DAF in VTP rabbits (DAF from RA; VTP rabbits without VTP. EPA rabbits; P = 0.004, DAF from LA; VTP rabbits vs. VTP + EPA rabbits; P = 0.003) (Fig. 2, B and C). AF inducibility by burst pacing was reduced from 35.0% to 7.52% by EPA treatment (Fig. 2D).

| Table 1. Echocardiographic measurements in rabbits treated with and without EPA |
|-----------------|-----------------|-----------------|-----------------|
|                 | VTP (< 15)      | VTP + EPA (< 15)|                 |
|                 | Baseline        | Postpacing      | Baseline        | Postpacing      |
| LAD, mm         | 7.6 ± 1.4       | 10.9 ± 2.4*     | 7.7 ± 1.1       | 10.9 ± 1.4*     |
| LVEDd, mm       | 15.1 ± 1.1      | 18.4 ± 2.1*     | 14.4 ± 1.0      | 18.4 ± 1.7*     |
| LVESd, mm       | 9.9 ± 1.1       | 15.9 ± 2.3*     | 9.1 ± 0.9       | 15.2 ± 1.9*     |
| IVSd, mm        | 2.9 ± 0.2       | 2.6 ± 0.4       | 2.6 ± 0.5       | 2.6 ± 0.7       |
| PWD, mm         | 2.7 ± 0.3       | 2.5 ± 0.5       | 2.6 ± 0.4       | 2.5 ± 0.5       |
| FS, %           | 34.6 ± 3.6      | 14.1 ± 4.4*     | 37.1 ± 3.7      | 17.4 ± 4.9*     |
| EF, %           | 67.5 ± 4.8      | 32.4 ± 8.9*     | 70.8 ± 4.7      | 38.6 ± 9.3*     |

Values are means ± SE. Baseline indicates state before pacing. *P < 0.05 vs. baseline, VTP, ventricular tachypacing; EPA, eicosapentaenoic acid; LAD, left atrial diameter; LVESd, left ventricular end-diastolic diameter; LVESd, left ventricular end-systolic diameter; IVSd, intraventricular septum diameter; PWD, posterior wall diameter of left ventricle; FS, fractional shortening; EF, ejection fraction.
tissues of rabbits were increased following VTP. EPA treatment significantly reduced collagen I and III mRNA levels in the atria (Fig. 3, C and D). VTP induced a significant increase in the expression of TGF-β1 in the rabbit atria, and this induction was inhibited by treatment with EPA (Fig. 3E). Activation of the ERK is an important mediator of atrial remodeling (5). Therefore, the phosphorylation of ERK in the LA tissues at day 7 after surgery was assessed by Western blot analysis. VTP resulted in a significant increase in ERK phosphorylation in the LA tissues, and the EPA treatment significantly attenuated the VTP-induced ERK phosphorylation (Fig. 3F).

Effect of EPA on the profiles of adipokines. Epicardial adipose tissue has recently been shown to play a role in the development of cardiovascular disease (35). Additionally, EPA exerts beneficial actions on the dysregulation of adipokine production (13, 25). Thus we assessed the role of adiponectin as an anti-inflammatory adipokine and TNF-α as a proinflammatory adipokine in epicardial adipose and LA tissue in each group. VTP did not affect mRNA levels of adiponectin in the epicardial adipose tissues of the rabbits. Treatment with EPA resulted in a 2.6-fold increase in this parameter independent of VTP (Fig. 4A). VTP did not affect protein expression of adiponectin in the rabbit atria. EPA treatment significantly increased adiponectin protein levels in the atria, (Fig. 4, C and D). In contrast, VTP resulted in a significant increase in TNF-α mRNA levels in the epicardial adipose tissues, and EPA treatment significantly attenuated VTP-induced mRNA levels of TNF-α (Fig. 4B). VTP significantly increased TNF-α protein levels in the rabbit atria. VTP-stimulated TNF-α expression in the rabbit LA was attenuated by EPA treatment (Fig. 4, C and E). EPA treatment thus modulated the profiles of adipokine production from epicardial adipose tissue. Finally, AMPK phosphorylation in the LA tissue was assessed by Western blotting, as adiponectin functions by inducing the AMPK phosphorylation (Fig. 3). VTP treatment significantly attenuated the VTP-induced ERK phosphorylation in the atria (Fig. 3, E). EPA treatment significantly reduced collagen I and III mRNA levels in the LA tissues of rabbits (Fig. 3, C and D). VTP induced a significant increase in the expression of TGF-β1 in the rabbit atria, and this induction was inhibited by treatment with EPA (Fig. 3E). Activation of the ERK is an important mediator of atrial remodeling (5). Therefore, the phosphorylation of ERK in the LA tissues at day 7 after surgery was assessed by Western blot analysis. VTP resulted in a significant increase in ERK phosphorylation in the LA tissues, and the EPA treatment significantly attenuated the VTP-induced ERK phosphorylation (Fig. 3F).

DISCUSSION

The present study provides the first experimental evidence that highly purified EPA, one class of PUFAs, prevents AF in a well-established HF animal model. Treatment of rabbit with EPA resulted in decreased DAF induced by burst pacing and atrial fibrosis, which was accompanied by reducing ERK activation and TGF-β1 expression in the atrium. EPA treatment also increased adiponectin as an anti-inflammatory adipokine and decreased TNF-α as a proinflammatory adipokine in the atrium and epicardial adipose tissues. Thus EPA, which was used clinically to treat hyperlipidemia, may be useful for prevention and treatment of AF associated with HF.

Recently, a number of experimental findings have shown that EPA has beneficial actions in the cardiovascular system by directly acting on the component cells such as cardiac myocytes in the heart and blood vessels (18, 33). Treatment with EPA reduced myocardial infarct size following ischemia-reperfusion in a rabbit model (23). EPA also ameliorated endothelial dysfunction in the aortas of diabetic rats (19). In in vitro experiments, EPA directly protected cardiac myocytes against...
hypoxia/reoxygenation-induced injury and inhibited endothelin-1-induced cardiomyocyte hypertrophy (10).

The occurrence and development of AF is associated with changes in cardiac structure known as structural atrial remodeling. Atrial structural remodeling refers to the activation of fibroblasts with increased fibrosis, resulting in heterogeneity of cardiac conduction tissue through the activation of various signaling pathways, including TGF-β (21). TGF-β is a key regulator of extracellular matrix synthesis. In fact, inhibition of TGF-β reduces myocardial fibrosis induced by aortic constriction and suppresses inflammatory responses in the heart and vessels (17). Recently, it was reported that EPA attenuates rat liver fibrosis and TGF-β expression caused by methionine- and choline-deficient diet. EPA is also shown to attenuate renal fibrosis and TGF-β expression in a diabetic mice model (44).

In the present study, treatment with EPA attenuated VTP-induced atrial fibrosis and expression of TGF-β1. Collectively, the ability of EPA to prevent atrial remodeling may be implicated in suppression of fibrosis through the inhibition of TGF-β1.

EPA treatment showed a slightly, but not significantly, improved conduction velocity after VTP, although we expected that the effects to reduce atrial fibrosis would be accompanied by an improvement in the conduction abnormalities associated with HF. The reason for this discrepancy may be explained by the small size of rabbit atria and the heterogeneity of fibrosis in our model. A heterogeneous spatial distribution of fibrosis at the LA posterior wall governing AF wave dynamics has been reported in HF sheep model (36). In addition, we measured the RAA to LAA activation times and then simply divided by the RAA-LAA distance. These methods for measuring conduction velocity were rather crude.

Unfortunately, it was technically difficult to analyze the activation pathway/wave front and mapping of the conduction path in rabbit atria. Studies of the cellular and molecular electrophysiology of this model would be of interest but are beyond the scope of the present study.

Another possible explanation for the beneficial effect of EPA on atrial structural remodeling may be that EPA modulates the profiles of adipokine production from epicardial adipose tissue. Epicardial fat is a metabolically active organ that generates a variety of bioactive molecules such as adiponectin and TNF-α, which could significantly affect cardiac remodeling (12). Of note, it has been reported that epicardial fat volume is highly associated with human AF (1). Among numerous adipokines, adiponectin is the only established adipokine with anti-inflammatory properties (24). EPA has been shown to increase the expression and secretion of adiponectin in adipocytes, leading to elevated levels of circulating adiponectin (13). The favorable effects of EPA on insulin resistance in obese rats are associated with an increase in the plasma concentration of adiponectin (25). We and other groups have reported that adiponectin protects against the development of cardiac remodeling under various pathological conditions (29). Suppression of adiponectin causes severe concentric cardiac hypertrophy in response to pressure overload (29). Treatment with adiponectin attenuates cardiac hypertrophy and fibrosis caused by angiotensin II infusion in mice (29). Adiponectin deficiency also contributes to impaired cardiac function following ischemia-reperfusion injury (30). In cultured cardiac myocytes, adiponectin stimulates phosphorylation of AMPK and suppresses agonist-stimulated ERK activation and hypertrophic response through its ability to activate the AMPK signaling (29). Consistent with these observations, the present
studies show that treatment with EPA stimulated the AMPK activation and attenuated the ERK phosphorylation in the heart following VTP, accompanied by elevated adiponectin expression in epicardial adipose tissue. Adiponectin inhibits agonist-stimulated TNF-α, an adipokine with proinflammatory properties in cardiac myocytes and macrophages (30). EPA also has been shown to decrease expression and secretion of TNF-α in adipocytes and macrophages (13). Therefore, the upregulation of adiponectin by EPA may at least in part contribute to the protection against atrial remodeling by perturbing the network of proinflammatory cytokines, including TNF-α. The relationship of epicardial adipose tissue to the adjacent myocardium...
could suggest paracrine regulation by this small fat depot although the relationship could not exclude systemic control. However, further detailed biochemical and genetic studies are required to clarify the causes and consequence relation between the upregulation of adiponectin by EPA and atrial remodeling.

In the present study, there were no significant differences between control and EPA-treated rabbits in the atrial size using echocardiography. Consistent with our observation, epicardial fat is highly associated with paroxysmal and persistent AF independent of traditional risk factors including left atrial enlargement (1). In contrast, several studies have shown a relationship between atrial size and AF risk (38). The durations of induced AF were short in our rabbit model, probably because of the small size of rabbit atria. Electrical mechanisms of AF may be also different because of the difference in heart rate, size of atria, and ionic channels expressed in the atrium between rabbit and human. Thus differences in species or experimental models could potentially explain these discrepancies.

The present study has several limitations. First, the detailed mechanism by which EPA alters AF substrate is unclear. Recent studies have reported that oxidant stress is associated with AF, and EPA has antioxidant effect in various cells (8, 14, 22, 44). We determined the effect of vitamins C and E using our rabbit tachypacing model because the antioxidant properties of both vitamins C and E are well recognized. Treatment with vitamins C and E did not affect the increase in DAF in VTP rabbits, consistent with previous report (Supplementary Fig. S1; supplemental material for this article is available online at the American Journal of Physiology Heart and Circulatory Physiology website) (34). Thus prevention of VTP-induced AF in rabbits did not share by antioxidant effect. Recent animal experimental studies have shown that treatment with statin or peroxisome proliferator-activated receptor-γ activator attenuates AF promotion, at least in part, through anti-inflammatory action (31, 34). EPA treatment also increased adiponectin as an anti-inflammatory adipokine and decreased TNF-α as a proinflammatory adipokine in the atrium. Collectively, the anti-inflammatory property of EPA may contribute to the attenuation of AF promotion in our rabbit model. However, further detailed biochemical studies using cellular electrophysiology are required for the better understanding of the precise mechanisms. Second, we determined the dosage as 300 mg/kg per day for the purpose of increasing the EPA level in plasma of rabbit. The dosage level in the present study was higher compared with the usual dosage in human. However, therapeutic approaches aimed at increasing EPA level in the blood stream could be beneficial for treatment of cardiovascular disease in human. Third, the durations of

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induced AF, even in VTP rabbits, were short, probably because of the small size of rabbit atria. However, DAF was reproducibly increased under VTP-induced HF conditions and reproducibly reduced by EPA treatment. Fourth, we did not use a proper control group with another PUFA showing specificity of EPA in the present study. Finally, we need to take care when our results are applied to clinical use because there are species differences between rabbit and human. Electrical mechanisms of AF may be different as a result of the difference in heart rate, size of atria, and ionic channels expressed in the atrium between rabbit and human.

In conclusion, our findings suggest that EPA treatment could suppress atrial arrhythmogenic structural remodeling, which is accompanied by improved adipokine regulation of the epicardial adipose tissue. The beneficial effect of EPA on atrial remodeling may contribute to the anti-AF potential in human.

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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the authors.

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