



## Original article

## Eicosapentaenoic acid reduces ischemic ventricular fibrillation via altering monophasic action potential in pigs

Ryuji Tsuburaya, Satoshi Yasuda\*, Yoshitaka Ito, Takashi Shiroto, Jun Yi Gao, Kenta Ito, Hiroaki Shimokawa

Department of Cardiovascular Medicine, Tohoku University Graduate School of Medicine, Sendai, Japan

## ARTICLE INFO

## Article history:

Received 14 January 2011  
 Received in revised form 13 May 2011  
 Accepted 23 May 2011  
 Available online 30 May 2011

## Keywords:

Fish oils  
 Sudden death  
 Ventricular arrhythmias  
 Ischemia  
 $K_{ATP}$  channel

## ABSTRACT

Although high intake of n-3 fatty acids is associated with reduced mortality of patients with ischemic heart disease, especially reduction in sudden cardiac death (SCD), the detailed mechanisms remain to be elucidated. Thus, the present study was designed to examine whether long-term treatment with eicosapentaenoic acid (EPA), a major component of n-3 fatty acids, reduces ischemia-induced ventricular fibrillation (VF) in pigs in vivo, and if so, what molecular mechanisms are involved. Male pigs were treated with either a control chow (control group) or a control chow plus EPA (600 mg/kg/day, PO, EPA group) for 3 weeks and were subjected to myocardial ischemia for 90 min ( $n = 8$  each) with measurement of the monophasic action potential (MAP), as a marker of ventricular electrophysiological activities. The EPA treatment significantly attenuated the occurrence of VF (control  $5.1 \pm 1.7$  vs. EPA  $1.5 \pm 0.8$  times/animal,  $P < 0.05$ ) and markedly reduced the mortality (control 50% vs. EPA 0%,  $P < 0.05$ ), with the attenuation of MAP duration shortening during ischemia (control  $-28.1 \pm 3.0\%$  vs. EPA  $-18.2 \pm 1.4\%$ ,  $P < 0.05$ ). These beneficial effects of EPA were abolished by pre-treatment with cromakalim, a  $K_{ATP}$  channel opener ( $0.3 \mu\text{g/kg/min}$ , IC). Furthermore, EPA significantly inhibited the mRNA and protein expression of Kir6.2, a major component of sarcolemmal  $K_{ATP}$  channels, in both the ischemic region and non-ischemic regions. These results indicate that long-term treatment with EPA reduces ischemia-induced VF and SCD in pigs in vivo, for which attenuation of MAP duration shortening may be involved.

© 2011 Elsevier Ltd. All rights reserved.

### 1. Introduction

Although the management of patients with acute myocardial infarction (AMI) has been markedly improved, more than 50% of those AMI patients still die prior to hospitalization [1]. Most pre-hospital deaths due to AMI occur within the first hour after the onset, mainly caused by ischemia-induced ventricular fibrillation (VF) but not by reperfusion arrhythmia [2]. Thus, it is critically important to develop an effective strategy to suppress ischemia-induced VF in the early phase of AMI in order to reduce sudden cardiac death (SCD).

Epidemiologic [3,4] and interventional studies [5,6] have shown that high intake of fish oil and long-chain n-3 polyunsaturated fatty acids (n-3 PUFA), including eicosapentaenoic acid (EPA, C20:5) and docosahexaenoic acid (DHA, C22:5), could reduce SCD. Importantly, the GISSI-Prevenzione trial demonstrated that long-term treatment with n-3 PUFA reduces SCD after AMI [7]. However, the detailed mechanisms for the inhibitory effects of n-3 PUFA on SCD and ischemia-induced VF have not been fully elucidated.

During myocardial ischemia, rapid activation of cardiac ATP-sensitive potassium channel ( $K_{ATP}$  channel) plays an important role in ischemia-induced VF via action potential shortening and heterogeneous ventricular repolarization [8,9]. EPA possesses several beneficial effects on the pathological processes of AMI, including inhibition of thrombus formation [10] and inflammation [11] and stimulation of endothelial production of nitric oxide [12]. In addition, the acute effect of EPA on the electrophysiological properties also was reported in the previous study [13], however, the long-term effects of EPA on ischemia-induced VF and SCD in vivo remain to be examined. In the present study, we demonstrated that long-term oral treatment with EPA reduces ischemia-induced VF and SCD in pigs in vivo through the attenuation of ischemia-induced action potential shortening, for which might be mediated by suppression of myocardial  $K_{ATP}$  channels.

### 2. Materials and methods

The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1996). All procedures were performed according to the protocols approved by the Institutional Committee for Use and Care of Laboratory Animals of Tohoku University (20Mda-46).

\* Corresponding author at: Department of Cardiovascular Medicine, Tohoku University Graduate School of Medicine, Seiryō-machi, Aoba-ku, Sendai 980-8574, Japan. Tel.: +81 22 717 7153; fax: +81 22 717 7156.

E-mail address: [syasuda@cardio.med.tohoku.ac.jp](mailto:syasuda@cardio.med.tohoku.ac.jp) (S. Yasuda).

### 2.1. Animals and EPA treatment

A total of 32 domestic male pigs (2–3 month-old and weighing 20–30 kg) were randomly divided into the following 2 groups; 16-pigs were orally given EPA (600 mg/kg/day, EPA ethyl ester of purity >99%, Mochida Pharmaceutical, Tokyo, Japan) for 21 days (EPA group), and the remaining 16-pigs were fed with a standard chow alone (control group). The present dose and duration of the EPA treatment were determined based on the previous study with rabbits [14]. The 30 kg body weight animals in the control group were fed only with the regular diet including 28 g fat, whereas a total fat intake in the EPA group was assessed to be 46 g.

### 2.2. Fatty acid analysis

The fatty acids composition of plasma, RBC, and homogenized heart tissue extracted from the interventricular septum (IVS) (100 mg of tissue/ml of saline) was determined by capillary gas chromatography [11]. Total lipids were extracted by Folch's procedure and then fatty acids were methylated with boron trifluoride and methanol, and then methylated fatty acids were analyzed using a gas chromatograph (Shimadzu GC-17A, Shimadzu Corporation, Kyoto, Japan) and a BPX70 capillary column (0.25 mm in internal diameter × 30 m in length, SGE International Ltd., Melbourne, Australia). Tricosanoic acid, C23:0 was used as an internal standard.

### 2.3. Porcine model of acute myocardial ischemia

After the 3-week treatment, the animals were anesthetized with ketamine hydrochloride (20 mg/kg, IM) and sodium pentobarbital (20 mg/kg/h, IV). Surface ECG, heart rate, and arterial blood pressure were continuously monitored by a polygraph recording system (LEG1000, Nihon-Kohden, Tokyo, Japan). We inserted a 7-Fr sheath into the left carotid artery for cardiac catheterization and a 9-Fr sheath into the right carotid artery for evaluation of monophasic action potential (MAP), which is a useful marker of cardiac electrical stability [15]. Then, myocardial ischemia in the territory of the left circumflex coronary artery (LCx) was induced by inflated a coronary angioplasty balloon catheter with a lowest pressure that completely occluded distal coronary flow. Global and regional left ventricular function during ischemia was assessed by left ventriculography under occluding the LCx (n = 4 each) [16]. For pulseless ventricular tachycardia (VT) or VF, direct current (DC) shock was delivered at 200, 300 and 360J in a stepwise manner. After 90 min of myocardial ischemia, the heart tissues were immediately extracted from the inter-ventricular septum (IVS) as a non-ischemic area and from the posterior wall (PW) of the left ventricle as an ischemic area, stored under –80 °C frozen by liquid nitrogen. The extent of the risk area relative to the left ventricle was evaluated by Evans blue staining (n = 4 each).

### 2.4. Measurement of monophasic action potential (MAP)

To evaluate the regional action potentials in vivo, MAP was measured as a useful marker of cardiac electrical stability [15]. MAP was recorded by 6-Fr MAP catheter (EP technologies, San Jose, CA). The MAP catheter was advanced into the LV under fluoroscopic guidance and recorded the MAP at the endocardial side of the LV by the contact electrode technique. First, MAP was measured at the IVS for evaluating electrical activities in the non-ischemic area and then moved toward the PW of the left ventricle to record MAP at the ischemic area throughout the experiment. MAP signals (amplified and filtered at a frequency of 0.05 to 200 Hz) were displayed on 8-channel PowerLab data acquisition system (AD Instruments Inc., Colorado, CO) simultaneously with ECG (Supplemental Figure 1). MAP was obtained under spontaneous heart beat after placement of the catheter electrode in a position that provided continuous and adequate recordings, when a stable amplitude, smooth

configuration and isopotential diastolic baseline (phase 4) persisted for at least 5 min. The QT interval, MAP duration at 90% repolarization (MAPD<sub>90</sub>) and at 50% repolarization (MAPD<sub>50</sub>) was measured, and collected for root square of RR interval (QTc, MAPD<sub>90c</sub> and MAPD<sub>50c</sub>) because these parameters highly depend on heart rate [17]. The upstroke velocity in phase 1 of MAP (dV/dt) was also calculated [18]. Chart5 (AD Instruments Inc., Colorado, CO) was used for analysis of MAP and ECG.

### 2.5. Intracoronary pre-treatment with cromakalim and 5-hydroxydecanoate

To evaluate the possible role of cardiac K<sub>ATP</sub> channel, additional 16-pigs (n = 8, each) were pre-treated with cromakalim (Sigma-Aldrich Inc., St Louis, MO), an agent selectively opens K<sub>ATP</sub> channel in the heart [19]. Cromakalim was dissolved in the vehicle (saline with polyethylene glycol and ethanol at a final concentration of <1%), selectively infused into the LCx, starting with 10 µg/kg bolus infusion at 10 min before myocardial ischemia followed by continuous infusion at 0.3 µg/kg/min until the end of the experiment. For selective drug administration into the ischemic LCx area, an infusion balloon catheter (Attendant®, Terumo Clinical Supply Co., Ltd, Gifu, Japan) was used, which enables to deliver drugs to the distal site while occluding the proximal LCx. To further evaluate the specific role of mitochondrial K<sub>ATP</sub> channel, additional 8-pigs were pre-treated with 5-hydroxydecanoate (5-HD; Sigma-Aldrich Inc., St Louis, MO), an agent selectively inhibits mitochondrial K<sub>ATP</sub> channel in the heart [20]. 5-HD was infused into the LCx for 45 min before myocardial ischemia followed by continuous infusion at 0.5 mg/kg/min until the end of the experiment. The present dose and duration of cromakalim or 5-HD were determined based on the previous study in dogs [21,22].

### 2.6. RT-PCR for cardiac K<sub>ATP</sub> channels

Total RNA was extracted using RNeasy Maxi kit (QIAGEN, Hilden, Germany) and 500 ng total RNA was reverse-transcribed using QuantiTect Reverse Transcription Kit (QIAGEN). Quantitative RT-PCR was performed for the 5 components of K<sub>ATP</sub> channel, including Kir6.1 (KCN8), Kir6.2 (KCNJ11), SUR1 (ABCC8), SUR2A and SUR2B (ABCC9), using a real-time detection system (Bio-Rad Lab, Hercules, CA). The following primers were used to amplify each component and GAPDH;

- KCNJ9* (forward, 5'-TGTTCCGTGTGGGGCGACTTA-3', and reverse, 5'-CAAGCGGGTTATCAACAGGAATG-3'),
- KCNJ8* (forward, 5'-GTGGTGAAACCACAGGCATC-3', and reverse, 5'-TGGTGTGCGCAACTTGAGTA-3'),
- ABCC8* (forward, 5'-TGCCGCACGTCTTCTACTCTTCA-3', and reverse, 5'-CAGGATGCCCTCTGCAATCTCACA-3'),
- ABCC9* (SUR2A: forward, 5'-CAGCTGAAGAATATGGTCAAATC-3', and reverse, 5'-CTACTTGTGGTCATCACCAAAG-3', SUR2B: forward, 5'-GCTTCCATTGACATGGCCACAGA-3', and reverse, 5'-GCCAAGAGGCTTTCTGGAGTGTA-3') and
- GAPDH* (forward, 5'-TGATGGGCATGAACCATGAGA-3', and reverse, 5'-TCCACGATGCCGAAGTTGTC-3').

*GAPDH*, *KCNJ8* and *KCNJ11* were custom designed (Takara Bio Inc., Shiga, Japan), based on the sequence of *Sus scrofa* publicly available on the web site of the National Center for Biotechnology Information. The sequence of *ABCC8* and *ABCC9* were based on the previous studies [23,24]. *GAPDH* was used as an internal control. SYBR Premix Ex Taq™ II (Takara Bio Inc.) was used for the detection of each components and *GAPDH* cDNA, respectively.

### 2.7. Western blot analysis for cardiac Kir6.2

To study further the inhibitory effect of EPA on Kir6.2 expression, western blot analysis for Kir6.2 was performed. The extracted samples

(20 µg of protein) were subjected to SDS-PAGE/immunoblot analysis by using the specific antibody for Kir6.2 (sc-11228, Santa Cruz Biotechnology, inc., CA). The regions containing proteins were visualized by electrochemiluminescence Western blotting luminal reagent (RPN2132, GE Healthcare UK Ltd., UK). The extents of Kir6.2 expression were normalized by that of GAPDH.

### 2.8. Statistical analysis

All results are expressed as mean ± SEM. The results of mortality were analyzed by Fisher's exact probability test. Unpaired Student's *t*-test was used for comparison of composition of fatty acids, arrhythmic events, MAP evaluation and the result of RT-PCR analysis. Statistical analyses were performed with SigmaStat for Windows version 3.00.0 (SPSS Inc, Chicago, IL). A value of *P*<0.05 was considered to be statistically significant.

## 3. Results

### 3.1. Effects of EPA treatment on fatty acids components

The long-term EPA treatment markedly increased the proportion of EPA (mol%) not only in plasma but also in RBC and cardiac tissue (all *P*<0.01) (Table 1). In contrast, the EPA treatment significantly decreased the proportion of arachidonic acid and DHA (mol%) in plasma, RBC and heart tissue (all *P*<0.01) (Table 1). The long term treatment with EPA also significantly increased the net concentration of EPA and decreased that of arachidonic acid and DHA, while the concentration of total fatty acids and other kinds of fatty acid including saturated fatty acids, oleic acids, linoleic acids and alpha-linoleic acids, did not change significantly in the 2 groups (Supplemental Table).

### 3.2. Inhibitory effect of EPA treatment on ischemia-induced VF in vivo

As reported previously [25], during myocardial ischemia, VF was frequently induced and was refractory to DC shocks in the control group, whereas VT was spontaneously terminated and was not sustained in the EPA group. The prevalence of pulseless VT/VF (EPA 1.5±0.8 vs. control 5.1±1.7 /animal, *P*<0.05), the DC shock delivery (EPA 393±202 vs. control 1653±590 J/animal, *P*<0.05) and the number of shocks applied was significantly reduced in the EPA group (EPA 1.5±0.8 vs. control 5.1±1.7/animal, *P*<0.05), while the DC shock intensity finally applied was comparable between the 2 groups (data not shown). All VF episodes in both groups were triggered by ventricular extra-systoles. In the control group, all of 41 pulseless VT/VF occurred within 15 min after coronary occlusion, whereas in the EPA group, the prevalence of pulseless VT/VF in this period was

42% (5 of 12 episodes) (Supplemental Fig. 2). As a result, the survival rate was significantly improved in the EPA group, while in the control group, half of the animals died due to VT/VF (survival rate: EPA 100% vs. control 50%, *P*<0.05).

During ischemia, myocardial blood flow in the LCx region (EPA 0.10±0.04 vs. control 0.10±0.08 ml/min/kg, *n*=4 each, measured by colored microspheres), left ventricular ejection fraction and regional wall motion was low and comparable between the 2 groups (Supplemental Figure 3). Evans blue staining demonstrated that the extent of the risk area relative to the LV was comparable between the 2 groups (EPA 38±2% vs. control 34±2%).

### 3.3. EPA treatment ameliorates ischemia-induced MAP shortening in vivo

At the baseline, heart rate (EPA 149±11 vs. control 140±8 beats/min), mean blood pressure (EPA 97±5 vs. control 99±5 mm Hg), QTc on surface ECG (EPA 455±10 vs. control 463±10 ms), MAPD<sub>90c</sub> in ischemic (EPA 384±11 vs. control 389±12 ms) and non-ischemic region (EPA 384±10 vs. control 387±11 ms), or MAPD<sub>50c</sub> in the ischemic (EPA 334±11 vs. control 320±9 ms) and the non-ischemic region (EPA 321±11 vs. control 329±9 ms) did not differ significantly between the 2 groups. The baseline MAP90c was comparable between the ischemic (387±8 ms) and non-ischemic (385±7 ms) region.

During the LCx occlusion, as compared with the baseline (= pre-ischemic) values, heart rate tended to increase in the control group (155±12 beats/min, *P*=0.06), whereas it did not change significantly in the EPA group (157±13 beats/min). Blood pressure did not change significantly in both groups (control group, 87±5 mm Hg; EPA group, 92±5 mm Hg). Neither post-ischemic heart rate nor blood pressures differed between the 2 groups.

Importantly, in the control group, MAPD<sub>90c</sub> at the ischemic region was significantly shortened within the first 5 min and reached to the steady state of 277±11 ms (−28.5±3.0% change from 387±11 ms at baseline) in 15 min (Fig. 1A), when VF occurred frequently (Supplemental Figure 2), whereas QTc and MAPD<sub>90c</sub> at the non-ischemic region did not change during ischemia (Fig. 1C). In contrast, in the EPA group, the ischemia-induced shortening of MAPD<sub>90c</sub> was significantly attenuated (315±11 ms, −18.1±1.4% change from 384±10 ms at baseline, *P*<0.05 vs. the control group) (Fig. 1B and 1C). Although MAPD<sub>50c</sub> at ischemic region significantly shortened during myocardial ischemia, there was no difference between the 2 groups (EPA; 193±12 ms, −41.8±3.6% change from baseline, control; 191±13 ms, −40.4±3.6% change from baseline). The dV/dt in phase 1 of MAP markedly decreased to a similar extent in the 2 groups during the ischemia (Fig. 1D).

### 3.4. Pre-treatment with cromakalim abolishes the beneficial effects of EPA in vivo

Before and after intracoronary administration of cromakalim, a K<sub>ATP</sub> channel opener, QTc on surface ECG did not change in the control (before 449±8 vs. after 448±8 ms) and the EPA group (before 452±10 vs. after 447±9 ms). Also, cromakalim pre-treatment did not significantly change heart rate or blood pressure before and during myocardial ischemia (data not shown). However, cromakalim abolished the inhibitory effects of EPA on ischemia-induced MAPD<sub>90</sub> shortening in the EPA group (EPA + cromakalim, −32.0±4.0%, *P*<0.05 compared with EPA alone), whereas cromakalim did not affect ischemia-induced shortening of MAPD<sub>90c</sub> in the control group (Fig. 2A–2C). Cromakalim also abolished the inhibitory effects of EPA on VT/VF occurrence (Fig. 2D) and deteriorated survival rate during ischemia in the EPA group (EPA + cromakalim 50%, *P*<0.05 compared with EPA alone). In the additional experiment, the pre-treatment with 5-HD, a selective mitochondrial K<sub>ATP</sub> channel blocker, tended to shorten MAPD<sub>90</sub> in the control group with a

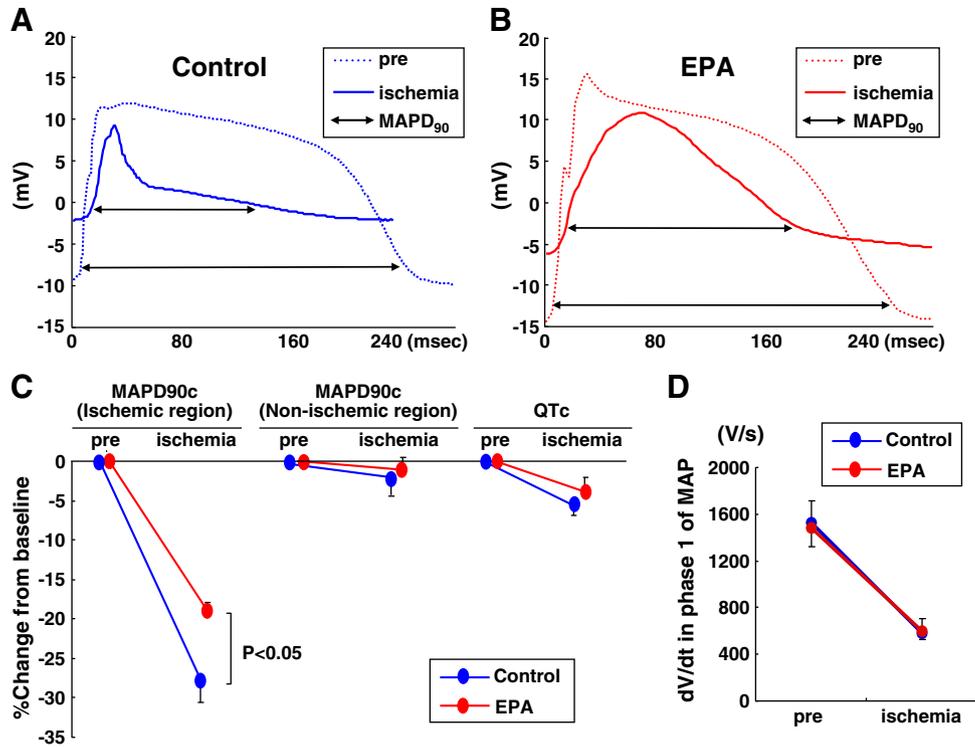
**Table 1**  
Fatty acids composition in the plasma, red blood cells and the heart.

	AA	EPA	DHA
<i>Plasma</i>			
Control (n=8)	6.88±0.68	0.38±0.06	1.18±0.18
EPA (n=8)	2.18±0.27*	11.38±1.13*	0.32±0.07*
<i>RBC</i>			
Control (n=8)	4.74±0.09	0.23±0.02	1.62±0.13
EPA (n=8)	2.68±0.11*	4.36±0.30*	1.10±0.06*
<i>Heart</i>			
Control (n=8)	14.32±0.40	1.30±0.18	1.34±0.13
EPA (n=8)	6.66±0.61*	13.96±0.67*	0.70±0.07*

Results (mol% to total fatty acids) are expressed as mean ± SEM.

AA, arachidonic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; RBC, red blood cells.

\**P*<0.01 vs. the control group.



**Fig. 1.** EPA treatment ameliorates ischemia-induced shortening of MAP. Representative MAP recordings in the control (A) and the EPA group (B) ( $n = 8$ , each). (C) The long-term EPA treatment ameliorated the ischemia-induced shortening of collected MAPD<sub>90</sub> (MAPD<sub>90c</sub>) at the ischemic region (left panel) but not at the non-ischemic region (middle panel) without alteration in collected QT (QTc) (right panel). (D) The upstroke velocity in phase 1 of MAP. Results are expressed as mean  $\pm$  SEM.

P-value of 0.06, which was not statistically significant (Supplemental Figure 4).

### 3.5. EPA suppresses cardiac expression of Kir6.2

RT-PCR analysis showed that among the 5 components of  $K_{ATP}$  channel, the EPA treatment significantly reduced cardiac mRNA expression of Kir6.2 (a major component of sarcolemmal  $K_{ATP}$  channel) and increased that of SUR2B (a major component of vascular smooth muscle cells), whereas other 3 components, Kir6.1, SUR1 and SUR2A, were unaffected by the EPA treatment (Fig. 3). Importantly, the EPA treatment also significantly reduced the cardiac protein expression of Kir6.2 (Fig. 4).

## 4. Discussion

The major findings of this study were that (1) the long-term EPA treatment ameliorated ischemia-induced VF and mortality, (2) ischemia-induced MAP shortening was significantly attenuated in the EPA group, (3) these beneficial effects of EPA were abolished by pre-treatment with cromakalim, a  $K_{ATP}$  channel opener, and (4) the EPA treatment significantly suppressed cardiac expression of Kir6.2, a major component of sarcolemmal  $K_{ATP}$  channel.

### 4.1. Validation of the present EPA treatment and comparison with previous study

In the present study, highly purified EPA was used. This may have advantage in comparison with the previous studies with fish oil or combination of EPA and DHA, which the usage formation, purity and dose of n-3 PUFA were too inhomogeneous to elucidate the exact anti-arrhythmic mechanism [26]. Indeed, fatty acids analysis showed that the EPA treatment of the present study markedly increased the concentra-

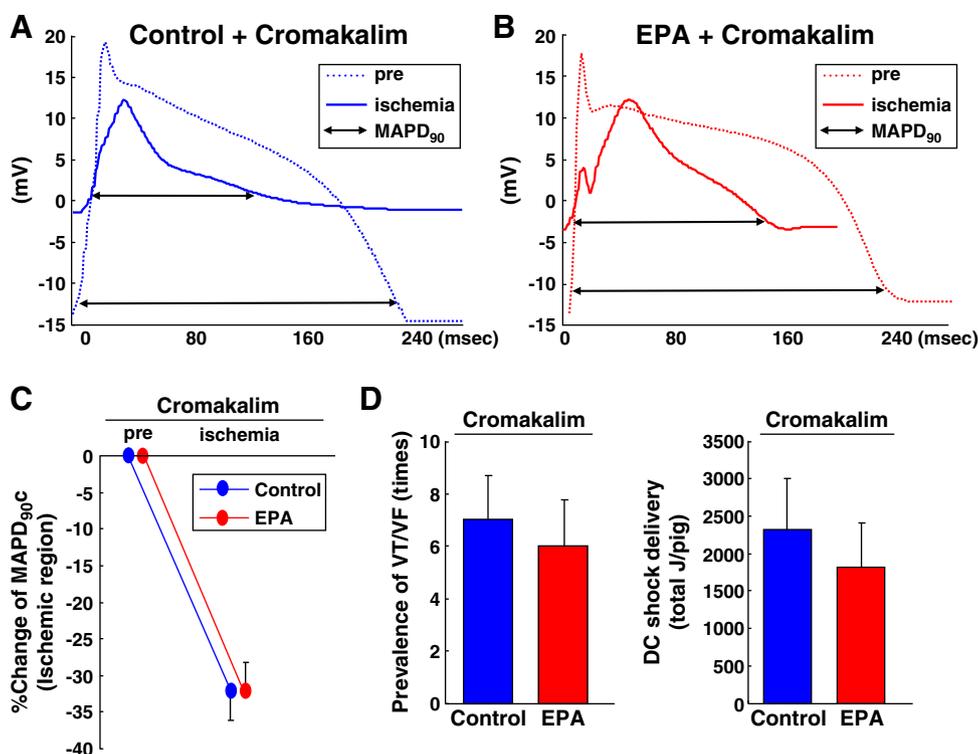
tion of EPA alone in the plasma, RBC and heart tissue, whereas it decreased the concentration of DHA, another major component of n-3 PUFA. It has been reported that the long-term treatment with EPA decreases DHA concentration which could be explained by the competitive inhibition by EPA of  $\Delta^6$ -desaturase, leading to DHA incorporation into the cell membrane [27].

The 30 kg body weight animals in the control group were only fed with the regular diet including 28 g fat, whereas a total fat intake was calculated to be 46 g in the EPA group. However, the concentration of total fatty acids and the other kinds of fatty acid including with saturated fatty acids, oleic acids, linoleic acids and alpha-linoleic acids did not change significantly in the 2 groups (Supplement Table). These results suggest that the non-specific effects of fatty acids may be minimal and that the present results with EPA were mediated by the increase in EPA.

### 4.2. Inhibitory effects of EPA on MAP shortening during myocardial ischemia

Pathogenesis of ischemia-induced ventricular arrhythmia is variable, depending on the time elapsed from onset of ischemia, typically classified to acute phase and delayed phase. Most pre-hospital deaths occur due to the acute phase arrhythmia within the first hour from onset of ischemia [2], whereas the delayed phase ventricular arrhythmia developing 6–72 h is associated with severe LV dysfunction and heart failure. In the present study, all VT/VF events in the control group occurred in the first 15 min following coronary artery occlusion (Supplemental Figure 2), and those acute phase fatal ventricular arrhythmias were suppressed by EPA treatment [25].

Previous ex vivo study with pigs receiving diets rich in fish oil for 8-weeks demonstrated that the number of ischemia-induced VT and VF increased in an experimental condition of isolated heart perfused with blood and Tyrodes solution and stimulated by pacing [28].



**Fig. 2.**  $K_{ATP}$  channel opener cromakalim abolishes the beneficial effects of EPA. Representative MAP recordings in the control (A) and the EPA group (B) ( $n = 8$ , each) following pre-treatment with intracoronary cromakalim infusion. The pre-treatment with cromakalim abolished the beneficial effects of EPA in terms of MAPD<sub>90c</sub> shortening (C), prevalence of VT/VF and DC shock delivery (D) ( $n = 8$ , each). Results are expressed as mean  $\pm$  SEM.

Although anti-arrhythmic effects of n-3 PUFA still remain controversial, accumulating clinical and experimental evidence indicates that patients with coronary artery disease are relatively sensitive to anti-SCD effect of n-3 PUFA [26]. In line with this notion, acute and chronic administration of n-3 PUFA actually decreases the frequency of ischemia-induced VT in vivo [29,30].

The changes in electrophysiological properties, such as ion channels and action potentials, following acute and chronic administration of n-3 PUFA were reported in vitro but in non-ischemic conditions [31,32]. Because these changes during myocardial ischemia remained to be elucidated, we measured MAP, which is well known to reflect transmembrane action potential of cardiomyocytes [33], and thought to be suitable for studying the local myocardial electrophysiology in vivo. Indeed, the continuous signal recording for several hours is available in MAP by contact electrode technique [34] and ischemia-induced shortening of action potential could be demonstrated when recording MAP in humans [35]. Also, the present finding on MAP (Supplemental Figure 1) is consistent with the previous study in pigs in vivo [36,37].

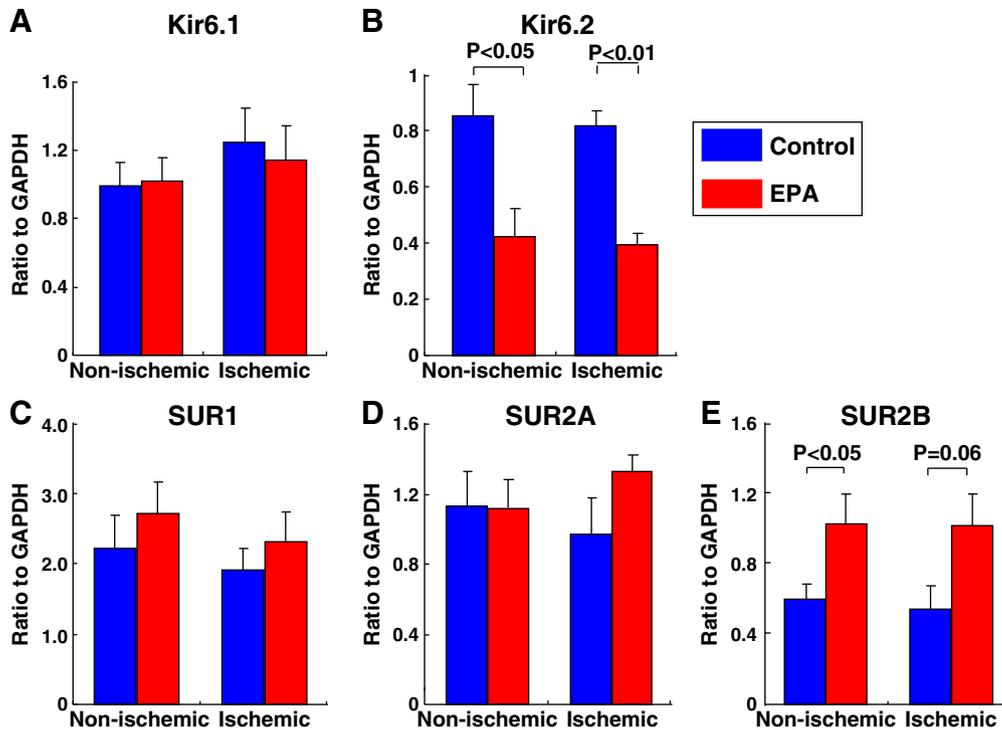
The present study clearly demonstrates a regional electrophysiological alteration that is otherwise undetectable by QT interval measurements on surface ECG. During the coronary occlusion, MAP was significantly shortened in 10–20 min at the ischemic region, whereas it remained unchanged at the non-ischemic region. Although the extent of area at risk was comparable between the 2 groups, the EPA treatment ameliorated the ischemia-induced shortening of MAPD<sub>90</sub> which reflects the total repolarization of the cardiomyocytes. Moreover, although previous studies showed that n-3 PUFA reduced adrenoceptor responses in experimental models [38] and heart rate in humans [39], the present EPA treatment did not significantly affect the hemodynamic parameters. These results indicate that the amelioration of ischemia-induced shortening of MAP duration plays an important role in the anti-arrhythmic effects of EPA.

#### 4.3. Potential involvement of cardiac $K_{ATP}$ channel inhibition in the anti-arrhythmic effects of EPA

$K_{ATP}$  channel activation in response to ATP depletion induced by hypoxia and myocardial ischemia plays a pivotal role in action potential shortening [40], although this response has been shown to elicit controversial results regarding arrhythmia triggering. Indeed, sarcolemmal  $K_{ATP}$  channel blockers, either non-selective (e.g. glibenclamide) or selective (HMR-1883), inhibit ischemia-induced action potential shortening and VF in a variety of experimental models [36,41], whereas non-selective antagonists of  $K_{ATP}$  channel (e.g., glibenclamide) could impair ventricular contraction and reduce coronary blood flow, leading to the development of malignant arrhythmias [42,43]. In the present study, intracoronary pre-treatment with  $K_{ATP}$  channel opener, cromakalim [19], abolished the beneficial effects of EPA on ischemia-induced shortening of MAP and tachyarrhythmia, whereas pre-treatment with the cromakalim did not increase the MAP shortening and arrhythmia in the control group. These results suggested that inhibiting cardiac  $K_{ATP}$  channel may contribute, at least in part, to the beneficial effects of EPA.

$K_{ATP}$  channel consists of a pore forming inward rectifying potassium channel (Kir6.1 or 6.2) and a regulatory sulfonylurea receptor (SUR1, SUR2A or SUR2B), and the combination of Kir and SUR determine the specificity of  $K_{ATP}$  channel. In particular, regarding the sarcolemmal membrane, Kir6.2 and SUR2A predominantly consist of the  $K_{ATP}$  channel [44]. The present result with the quantitative real time RT-PCR and western blot analysis showed that the long-term EPA treatment down-regulates sarcolemmal Kir6.2 both in the ischemic region and non-ischemic region. Interestingly, the EPA treatment did not significantly affect the expression of SUR2A, a target of the  $K_{ATP}$  channel blockers (glibenclamide and HMR-1883) [45].

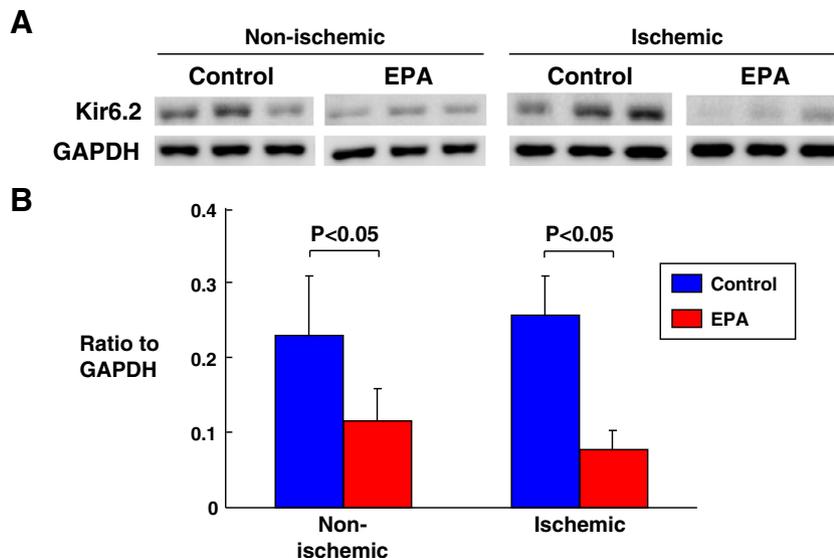
Careful interpretation is needed regarding the pharmacological finding that the EPA effects were abolished by pre-treatment with a non-specific  $K_{ATP}$  channel opener cromakalim. In the case of the



**Fig. 3.** EPA treatment suppresses myocardial mRNA expression of Kir6.2. The long-term treatment with EPA significantly suppressed myocardial mRNA expression of Kir6.2 (major component of sarcolemmal  $K_{ATP}$  channel), had no effect on that of Kir6.1, SUR1, or SUR2A, and rather enhanced that of SUR2B (a major component of vascular smooth muscle cells) in both the ischemic and non-ischemic regions ( $n = 8$ , each). Results are expressed as mean  $\pm$  SEM.

downregulation of Kir6.2 expression, the effect of cromakalim on MAP is unlikely to be associated mainly with sarcolemmal  $K_{ATP}$  channel activation. Although the selective mitochondrial  $K_{ATP}$  channel blocker, 5-HD did not clearly mimic the EPA effects (Supplemental Figure 4), the involvement of mitochondrial  $K_{ATP}$  channels, which are also activated by cromakalim, might be considered as a potential site of cardioprotection and anti-arrhythmic activity. Further studies are needed to determine by which mechanism EPA could cause downregulation of Kir6.2 and whether intracoronary administration of cromakalim could alter Kir6.2 protein expression.

In the present study, the EPA treatment did not affect the expression of Kir6.1, a Kir component in the vascular smooth muscle cells [46] and mitochondria [47], and of SUR1, a potential candidate of SUR component for mitochondria [48]. However, the expression of SUR2B, a main component in vascular smooth muscle cells [46] was significantly increased in the EPA-treated group. Taken together with the previous finding of  $K_{ATP}$ -mediated, endothelium-independent vasodilatation by EPA of isolated rat aorta [49], the increased expression of SUR2B by EPA could be beneficial to ameliorate myocardial ischemia.



**Fig. 4.** EPA treatment suppresses myocardial protein expression of Kir6.2. The long-term treatment with EPA significantly suppressed myocardial protein expression of Kir6.2 (major component of sarcolemmal  $K_{ATP}$  channel) in both the ischemic and non-ischemic regions ( $n = 8$ , each). Results are expressed as mean  $\pm$  SEM.

#### 4.4. Limitation of the study

Several limitations of the present study should be mentioned. First, the present study was performed in normal juvenile pigs without preexisting atherosclerotic coronary plaques or myocardial dysfunction, which could affect myocardial responses to acute ischemia. Indeed, the long-term treatment with EPA reduces major coronary events in high-risk patients in the Japan EPA Lipid Intervention Study (JELIS) [50]. Second, the direct evidence for inhibition of  $K_{ATP}$  function by the EPA treatment needs to be demonstrated in a future study (e.g. patch clamp study with isolated cardiomyocytes). Third, we could not exclude the possible effects of lower AA and DHA levels in the EPA treated animals. Fourth, it has been reported that EPA inhibits cardiac  $Na^+$  channels and L-type  $Ca^{2+}$  channels [13,31]. Although the EPA treatment did not affect the upstroke velocity in phase 1 and  $MAPD_{50}$  [51], these possible involvements were not fully investigated in the present study.

#### 4.5. Conclusion

In conclusion, the present study indicate that long-term treatment with EPA suppresses ischemia-induced VF and SCD in pigs in vivo, for which improvement of MAP shortening may be involved. These results may account, at least in part, for the beneficial inhibitory effects of EPA on SCD in ischemic heart disease.

#### Conflict of interest statement

None declared.

#### Acknowledgments

The authors' work was supported in part by the grants-in-aid [18890018] from the Scientific Research; and the global COE project [F02] from the Japanese Ministry of Education, Culture, Sports, Science, and Technology, Tokyo, Japan. The authors thank Dr. Teruyuki Yanagisawa (Department of Molecular Pharmacology, Tohoku University Graduate School of Medicine), Dr. Masaaki Soma (Mochida Pharmaceutical Co., Ltd.) and Dr. Takatoshi Hizume (Kyushu University Graduate School of Medicine) for their valuable cooperation in the present study and Mochida Pharmaceutical Co., Ltd. for providing the EPA.

#### Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.jmcc.2011.05.018.

#### References

- [1] Lowel H, Dobson A, Keil U, Herman B, Hobbs MS, Stewart A, et al. Coronary heart disease case fatality in four countries. A community study. The Acute Myocardial Infarction Register Teams of Auckland, Augsburg, Bremen, FINMONICA, Newcastle, and Perth. *Circulation* 1993;88:2524–31.
- [2] O'Doherty M, Tayler DI, Quinn E, Vincent R, Chamberlain DA. Five hundred patients with myocardial infarction monitored within one hour of symptoms. *Br Med J (Clin Res Ed)* 1983;286:1405–8.
- [3] Bang HO, Dyerberg J, Hjorne N. The composition of food consumed by Greenland Eskimos. *Acta Med Scand* 1976;200:69–73.
- [4] Albert CM, Campos H, Stampfer MJ, Ridker PM, Manson JE, Willett WC, et al. Blood levels of long-chain n-3 fatty acids and the risk of sudden death. *N Engl J Med* 2002;346:1113–8.
- [5] Burr ML, Fehily AM, Gilbert JF, Rogers S, Holliday RM, Sweetnam PM, et al. Effects of changes in fat, fish, and fibre intakes on death and myocardial reinfarction: diet and reinfarction trial (DART). *Lancet* 1989;2:757–61.
- [6] Leaf A, Albert CM, Josephson M, Steinhaus D, Kluger J, Kang JX, et al. Prevention of fatal arrhythmias in high-risk subjects by fish oil n-3 fatty acid intake. *Circulation* 2005;112:2762–8.
- [7] GISSI-Prevenzione Investigators. Dietary supplementation with n-3 polyunsaturated fatty acids and vitamin E after myocardial infarction: results of the GISSI-Prevenzione trial. Gruppo Italiano per lo Studio della Sopravvivenza nell'Infarto miocardico. *Lancet* 1999;354:447–55.
- [8] Venkatesh N, Lamp ST, Weiss JN. Sulfonylureas, ATP-sensitive  $K^+$  channels, and cellular  $K^+$  loss during hypoxia, ischemia, and metabolic inhibition in mammalian ventricle. *Circ Res* 1991;69:623–37.
- [9] Chi L, Uprichard AC, Lucchesi BR. Proarrhythmic actions of pinacidil in a conscious canine model of sudden coronary death. *J Cardiovasc Pharmacol* 1990;15:452–64.
- [10] Hirai A, Terano T, Hamazaki T, Sajiki J, Kondo S, Ozawa A, et al. The effects of the oral administration of fish oil concentrate on the release and the metabolism of [ $^{14}C$ ]arachidonic acid and [ $^{14}C$ ]eicosapentaenoic acid by human platelets. *Thromb Res* 1982;28:285–98.
- [11] Matsumoto M, Sata M, Fukuda D, Tanaka K, Soma M, Hirata Y, et al. Orally administered eicosapentaenoic acid reduces and stabilizes atherosclerotic lesions in ApoE-deficient mice. *Atherosclerosis* 2008;197:524–33.
- [12] Tagawa H, Shimokawa H, Tagawa T, Kuroiwa-Matsumoto M, Hirooka Y, Takeshita A. Long-term treatment with eicosapentaenoic acid augments both nitric oxide-mediated and non-nitric oxide-mediated endothelium-dependent forearm vasodilation in patients with coronary artery disease. *J Cardiovasc Pharmacol* 1999;33:633–40.
- [13] Leaf A, Kang JX, Xiao YF, Billman GE. Clinical prevention of sudden cardiac death by n-3 polyunsaturated fatty acids and mechanism of prevention of arrhythmias by n-3 fish oils. *Circulation* 2003;107:2646–52.
- [14] Ogita H, Node K, Asanuma H, Sanada S, Takashima S, Minamino T, et al. Eicosapentaenoic acid reduces myocardial injury induced by ischemia and reperfusion in rabbit hearts. *J Cardiovasc Pharmacol* 2003;41:964–9.
- [15] Franz MR. Long-term recording of monophasic action potentials from human endocardium. *Am J Cardiol* 1983;51:1629–34.
- [16] Sheehan FH, Bolson EL, Dodge DG, Schofer J, Woo HW. Advantages and applications of the centerline method for characterizing regional ventricular function. *Circulation* 1986;74:293–305.
- [17] Osaka T, Yokoyama E, Kushiyama Y, Hasebe H, Kuroda Y, Suzuki T, et al. Opposing effects of bepridil on ventricular repolarization in humans. Inhomogeneous prolongation of the action potential duration vs flattening of its restitution kinetics. *Circ J* 2009;73:1612–8.
- [18] Levine JH, Moore EN, Kadish AH, Guarnieri T, Spear JF. The monophasic action potential upstroke: a means of characterizing local conduction. *Circulation* 1986;74:1147–55.
- [19] Escande D, Thuringer D, Leguern S, Caverio I. The potassium channel opener cromakalim (BRL 34915) activates ATP-dependent  $K^+$  channels in isolated cardiac myocytes. *Biochem Biophys Res Commun* 1988;154:620–5.
- [20] Jaburek M, Yarov-Yarovoy V, Pauczek P, Garlid KD. State-dependent inhibition of the mitochondrial  $K_{ATP}$  channel by glyburide and 5-hydroxydecanoate. *J Biol Chem* 1998;273:13578–82.
- [21] Grover GJ, D'Alonzo AJ, Parham CS, Darbenzio RB. Cardioprotection with the  $K_{ATP}$  opener cromakalim is not correlated with ischemic myocardial action potential duration. *J Cardiovasc Pharmacol* 1995;26:145–52.
- [22] Sanada S, Kitakaze M, Asanuma H, Harada K, Ogita H, Node K, et al. Role of mitochondrial and sarcolemmal K(ATP) channels in ischemic preconditioning of the canine heart. *Am J Physiol Heart Circ Physiol* 2001;280:H256–63.
- [23] Ploug KB, Sorensen MA, Strobeck L, Klaerke DA, Hay-Schmidt A, Sheykhzade M, et al.  $K_{ATP}$  channels in pig and human intracranial arteries. *Eur J Pharmacol* 2008;601:43–9.
- [24] Kajioaka S, Nakayama S, Asano H, Seki N, Naito S, Brading AF. Levromakalim and MgGDP activate small conductance ATP-sensitive  $K^+$  channels of  $K^+$  channel pore 6.1/sulfonylurea receptor 2A in pig detrusor smooth muscle cells: uncoupling of cAMP signal pathways. *J Pharmacol Exp Ther* 2008;327:114–23.
- [25] Yasuda S, Shimokawa H. Acute myocardial infarction: the enduring challenge for cardiac protection and survival. *Circ J* 2009;73:2000–8.
- [26] Leon H, Shibata MC, Sivakumaran S, Dorgan M, Chatterley T, Tsuyuki RT. Effect of fish oil on arrhythmias and mortality: systematic review. *BMJ* 2008;337:a2931.
- [27] Mori TA, Burke V, Puddey IB, Watts GF, O'Neal DN, Best JD, et al. Purified eicosapentaenoic and docosahexaenoic acids have differential effects on serum lipids and lipoproteins, LDL particle size, glucose, and insulin in mildly hyperlipidemic men. *Am J Clin Nutr* 2000;71:1085–94.
- [28] Coronel R, Wilms-Schopman FJ, Den Ruijter HM, Belterman CN, Schumacher CA, Opthof T, et al. Dietary n-3 fatty acids promote arrhythmias during acute regional myocardial ischemia in isolated pig hearts. *Cardiovasc Res* 2007;73:386–94.
- [29] Billman GE, Hallaq H, Leaf A. Prevention of ischemia-induced ventricular fibrillation by omega 3 fatty acids. *Proc Natl Acad Sci USA* 1994;91:4427–30.
- [30] Kinoshita I, Itoh K, Nishida-Nakai M, Hirota H, Otsuji S, Shibata N. Antiarrhythmic effects of eicosapentaenoic acid during myocardial infarction—enhanced cardiac microosomal (Ca $^{2+}$ -Mg $^{2+}$ -ATPase activity). *Jpn Circ J* 1994;58:903–12.
- [31] Verkerk AO, van Ginneken AC, Berecki G, den Ruijter HM, Schumacher CA, Veldkamp MW, et al. Incorporated sarcolemmal fish oil fatty acids shorten pig ventricular action potentials. *Cardiovasc Res* 2006;70:509–20.
- [32] Kang JX, Xiao YF, Leaf A. Free, long-chain, polyunsaturated fatty acids reduce membrane electrical excitability in neonatal rat cardiac myocytes. *Proc Natl Acad Sci USA* 1995;92:3997–4001.
- [33] Franz MR, Burkhoff D, Spurgeon H, Weisfeldt ML, Lakatta EG. In vitro validation of a new cardiac catheter technique for recording monophasic action potentials. *Eur Heart J* 1986;7:34–41.
- [34] Franz MR. Current status of monophasic action potential recording: theories, measurements and interpretations. *Cardiovasc Res* 1999;41:25–40.
- [35] Taggart P, Sutton PM, Boyett MR, Lab M, Swanton H. Human ventricular action potential duration during short and long cycles. Rapid modulation by ischemia. *Circulation* 1996;94:2526–34.

- [36] Wirth KJ, Uhde J, Rosenstein B, Englert HC, Gogelein H, Scholkens BA, et al. K (ATP) channel blocker HMR 1883 reduces monophasic action potential shortening during coronary ischemia in anesthetised pigs. *Naunyn Schmiedebergs Arch Pharmacol* 2000;361:155–60.
- [37] Geelen P, O'Hara GE, Plante S, Philippon F, Gilbert M, Turgeon J. Ischemia-induced action potential shortening is blunted by d-sotalol in a pig model of reversible myocardial ischemia. *J Cardiovasc Pharmacol* 2000;35:638–45.
- [38] Reibel DK, Holahan MA, Hock CE. Effects of dietary fish oil on cardiac responsiveness to adrenoceptor stimulation. *Am J Physiol* 1988;254:H494–9.
- [39] Mozaffarian D, Geelen A, Brouwer IA, Geleijnse JM, Zock PL, Katan MB. Effect of fish oil on heart rate in humans: a meta-analysis of randomized controlled trials. *Circulation* 2005;112:1945–52.
- [40] Gasser RN, Vaughan-Jones RD. Mechanism of potassium efflux and action potential shortening during ischaemia in isolated mammalian cardiac muscle. *J Physiol* 1990;431:713–41.
- [41] Vajda S, Baczkó I, Lepran I. Selective cardiac plasma-membrane K(ATP) channel inhibition is defibrillatory and improves survival during acute myocardial ischemia and reperfusion. *Eur J Pharmacol* 2007;577:115–23.
- [42] Lu L, Reiter MJ, Xu Y, Chicco A, Greyson CR, Schwartz GG. Thiazolidinedione drugs block cardiac  $K_{ATP}$  channels and may increase propensity for ischemic ventricular fibrillation in pigs. *Diabetologia* 2008;51:675–85.
- [43] del Valle HF, Lascano EC, Negroni JA, Crottogini AJ. Glibenclamide effects on reperfusion-induced malignant arrhythmias and left ventricular mechanical recovery from stunning in conscious sheep. *Cardiovasc Res* 2001;50:474–85.
- [44] Inagaki N, Gono T, Clement JP, Wang CZ, Aguilar-Bryan L, Bryan J, et al. A family of sulfonylurea receptors determines the pharmacological properties of ATP-sensitive  $K^+$  channels. *Neuron* 1996;16:1011–7.
- [45] Moreau C, Jacquet H, Prost AL, D'Hahan N, Vivaudou M. The molecular basis of the specificity of action of K(ATP) channel openers. *EMBO J* 2000;19:6644–51.
- [46] Yamada M, Isomoto S, Matsumoto S, Kondo C, Shindo T, Horio Y, et al. Sulphonylurea receptor 2B and Kir6.1 form a sulphonylurea-sensitive but ATP-insensitive  $K^+$  channel. *J Physiol* 1997;499(Pt 3):715–20.
- [47] Suzuki M, Kotake K, Fujikura K, Inagaki N, Suzuki T, Gono T, et al. Kir6.1: a possible subunit of ATP-sensitive  $K^+$  channels in mitochondria. *Biochem Biophys Res Commun* 1997;241:693–7.
- [48] Liu Y, Ren G, O'Rourke B, Marban E, Seharaseyon J. Pharmacological comparison of native mitochondrial K(ATP) channels with molecularly defined surface K(ATP) channels. *Mol Pharmacol* 2001;59:225–30.
- [49] Engler MB, Engler MM, Browne A, Sun YP, Sievers R. Mechanisms of vasorelaxation induced by eicosapentaenoic acid (20:5n-3) in WKY rat aorta. *Br J Pharmacol* 2000;131:1793–9.
- [50] Matsuzaki M, Yokoyama M, Saito Y, Origasa H, Ishikawa Y, Oikawa S, et al. Incremental effects of eicosapentaenoic acid on cardiovascular events in statin-treated patients with coronary artery disease. *Circ J* 2009;73:1283–90.
- [51] Schouten VJ. The negative correlation between action potential duration and force of contraction during restitution in rat myocardium. *J Mol Cell Cardiol* 1986;18:1033–45.