Impact of isorhamnetin for suppression of electrical and structural remodeling via CaMKII-RyR2 and TRPC-mediated MAPK pathways in atrial fibrillation

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Abstract

Background and Purpose: Isorhamnetin, a natural flavonoid, has strong antioxidant and anti-fibrotic effects, and a regulatory effect against Ca2+-handling. Atrial remodeling due to fibrosis and abnormal intracellular Ca2+ activities contributes to initiation and persistence of atrial fibrillation (AF). This study investigated the effect of isorhamnetin on angiotensin II (AngII)-induced AF in mice. Experimental Approach: Wild-type male mice (C57BL/6J, 8 weeks old) were assigned to three groups: (1) control group, (2) AngII-treated group, and (3) AngII-and isorhamnetin-treated groups. AngII (1000 ng/kg/min) and isorhamnetin (5 mg/kg) were administered continuously via an implantable osmotic pump for two weeks and intraperitoneally one week before initiating AngII administration, respectively. AF induction and electrophysiological studies, Ca2+ imaging with isolated atrial myocytes and HL-1 cells, and action potential duration (APD) measurements using HL-1 cells were performed. AF-related molecule expression was assessed and histopathological examination was performed. Key Results: Isorhamnetin decreased AF inducibility compared to the AngII group and restored AngII-induced atrial effective refractory period prolongation. Isorhamnetin eliminated abnormal diastolic intracellular Ca2+ activities induced by AngII. Isorhamnetin also abrogated AngII-induced APD prolongation and abnormal Ca2+ loading in HL-1 cells. Furthermore, isorhamnetin strongly attenuated AngII-induced left atrial enlargement and atrial fibrosis. AngII-induced elevated expression of AF-associated molecules, such as ox-CaMKII, p-RyR2, p-JNK, p-ERK, and TRPC3/6, was improved by isorhamnetin treatment. Conclusion and Implications: The findings of this study suggest that isorhamnetin prevents AngII-induced AF vulnerability and arrhythmogenic atrial remodeling via modulating CaMKII-RyR2 and TRPC-mediated MAPK pathways, highlighting its potential as an anti-arrhythmogenic pharmaceutical or dietary supplement.

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Short running title: Novel therapeutic impact of isorhamnetin for atrial fibrillation

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Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request. Some data may not be made available because of privacy or ethical restrictions.

Author contribution statement

K.A., D.X., N.M., and H.I. designed the study. K.A., D.X., Y.O., Z.Y., S.L., Y.M., and K.T. participated in the data acquisition and analysis. K.T. and K.A. synthesized the isorhamnetin for the experiments. H.I., K.T., A.N., M.I., and K.A. supervised this study. K.A. and D.X. drafted the manuscript. H.I., K.T., K.A., and N.M. revised the manuscript for intellectual content.

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Conflict of interest

The authors have declared that no conflict of interest exists.

Abstrac t

*Background and Purpose***:** Isorhamnetin, a natural flavonoid, has strong antioxidant and anti-fibrotic effects, and a regulatory effect against Ca^{2+}-handling. Atrial remodeling due to fibrosis and abnormal intracellular Ca^{2+} activities contributes to initiation and persistence of atrial fibrillation (AF). This study investigated the effect of isorhamnetin on angiotensin II (AngII)-induced AF in mice.

*Experimental Approach***:** Wild-type male mice (C57BL/6J, 8 weeks old) were assigned to three groups: (1) control group, (2) AngII-treated group, and (3) AngII-and isorhamnetin-treated groups. AngII (1000 ng/kg/min) and isorhamnetin (5 mg/kg) were administered continuously via an implantable osmotic pump for two weeks and intraperitoneally one week before initiating AngII administration, respectively. AF induction and electrophysiological studies, Ca^{2+} imaging with isolated atrial myocytes and HL-1 cells, and action potential duration (APD) measurements using HL-1 cells were performed. AF-related molecule expression was assessed and histopathological examination was performed.

*Key Results***:** Isorhamnetin decreased AF inducibility compared to the AngII group and restored AngII-induced atrial effective refractory period prolongation. Isorhamnetin eliminated abnormal diastolic intracellular Ca^{2+} activities induced by AngII. Isorhamnetin also abrogated AngII-induced APD prolongation and abnormal Ca^{2+}loading in HL-1 cells. Furthermore, isorhamnetin strongly attenuated AngII-induced left

atrial enlargement and atrial fibrosis. AngII-induced elevated expression of AF-associated molecules, such as ox-CaMKII, p-RyR2, p-JNK, p-ERK, and TRPC3/6, was improved by isorhamnetin treatment.

Conclusion and Implications: The findings of this study suggest that isorhamnetin prevents AngII-induced AF vulnerability and arrhythmogenic atrial remodeling via modulating CaMKII-RyR2 and TRPC-mediated MAPK pathways, highlighting its potential as an anti-arrhythmogenic pharmaceutical or dietary supplement.

Keywords

isorhamnetin, atrial fibrillation, CaMKII, MAPK, TRPC, Ca^{2+}-handling

1.INTRODUCTION

Atrial fibrillation (AF) is the most common arrhythmia and is associated with substantial morbidity and mortality. The clinical burden of AF has been growing in recent years and is predicted to continue increasing in the future (Williams et al., 2020). Although pulmonary vein isolation is recommended as first-line therapy for patients with symptomatic AF, there are no established preventive therapies (Calkins et al., 2017). Conventional antiarrhythmic drugs that target ion channels in the heart have limitations in terms of their efficacy and side effects, and novel therapeutic targets are needed. Preventive upstream therapy for AF remains a significant, unmet medical need (Nattel et al., 2021).

At least two major pathophysiological mechanisms contribute to AF development: electrical and structural remodeling (Nattel & Harada, 2014). As a process of electrical remodeling, one of the pathogenic mechanisms of AF is the activation of Ca/calmodulin-dependent protein kinase II (CaMKII), which phosphorylates serin 2814 on ryanodine receptor 2 (RyR2), ultimately contributing to increased sarcoplasmic reticulum (SR) Ca^{2+} leakage (Wang et al., 2018). On the other hand, it has been demonstrated that morphological changes, especially interstitial fibrosis, play an important role in the substrate of AF (He et al., 2011). Atrial fibrosis occurs mainly because of the complex activity of various molecules, such as the mitogen-activated protein kinase (MAPK) family and transforming growth factor beta (TGFβ) (Nattel et al., 2020). These alterations are known as structural remodeling. Transient receptor potential (TRP) channels modulate Ca^{2+} entry and links the response of cardiac fibroblasts to remodeling stimuli that cause arrhythmias (Rose et al., 2012). Among them, TRP canonical-3 (TRPC3) and TRP canonical-6 (TRPC6) play a major role in cardiac hypertrophy and fibrosis; TRPC3/TRPC6 contributes to the regulation of fibroblast function via the extracellular signal-regulated kinase (ERK) and c-Jun N-terminal kinase (JNK) pathways, respectively (Harada et al., 2012; Nishida et al., 2007). In summary, modulating electrophysiological functions and improving morphological abnormalities in atrial tissue could be a promising therapeutic strategy for decreasing the initiation of AF.

Isorhamnetin, also known as 3'-methoxyquercetin, is a natural flavonoid commonly found in some plant-derived foods, such as wine and nuts, and is an immediate metabolite of quercetin. We have previously reported that isorhamnetin prevents fibrosis, hypertrophy, and inflammation in an angiotensin (AngII)-infused mouse model and reduces liver fibrosis and hepatic steatosis in non-alcoholic steatohepatitis mouse models by inhibiting the TGFβ pathways (Aonuma et al., 2020; Ganbold et al., 2019). Furthermore, isorhamnetin has been reported to have the ability to regulate vascular Ca^{2+}channels and currents (Saponara et al., 2011; Zhu et al., 2005). These findings suggest that flavonoids can be used as therapeutic targets to ameliorate electrophysiology-related diseases. Moreover, flavonoids have recently been reported to reduce the risk of AF development in high-risk patients in clinical practice; however, detailed reports exploring the mechanisms of this effect are lacking (Bondonno et al., 2020).

This study aimed to investigate the impact of isorhamnetin on AF vulnerability and explore its potential pathways.

2. METHODS

2.1 Animal preparation

All animal experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals of the US National Institutes of Health, the Animal Experiment Regulations of the University of Tsukuba, and the Basic Guidelines for the Proper Conduct of Animal Experiments in Academic Research Institutions under the jurisdiction of the Ministry of Education, Culture, Sports, Science, and Technology of Japan. Animal experiments were performed with approval from the University of Tsukuba Animal Experiment Committee.

Male C57BL/6 mice (eight weeks old; Japan Charles River Kanagawa, Japan) were randomly assigned to three groups: control, AngII-infused (AngII), and AngII and isorhamnetin-treated (AngII+isorhamnetin). AngII (1,000 ng/kg/h) was administered continuously for two weeks using an osmotic minipump (Alzet, model 2002; Cupertino, United States). Normal saline was injected into the mini-osmotic pump in the control group. Isorhamnetin (5 mg/kg) was intraperitoneally administered daily for three weeks to mice in the AngII+isorhamnetin group one week prior to AngII administration. Isorhamnetin was suspended in 0.1% dimethyl sulfoxide (DMSO) and 1% polypropylene glycol. In addition, the same amount of vehicle solution (0.1% dimethyl sulfoxide, 1% polypropylene glycol, and saline) was administered intraperitoneally as a sham injection to the mice in the control and AngII groups. After three weeks, the mice were euthanized by CO_2 inhalation and their hearts were harvested for further experiments. The mice used to obtain biochemical and histological data were not assigned to the AF induction study to avoid causing mechanical effects associated with catheters.

2.2 HL-1 cell culture

HL-1 mouse atrial myocytes were maintained in Claycomb medium supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin, and 100 μM norepinephrine in 30 mM L-ascorbic acid, 2 mM L-glutamine, and 10% FBS at 37°C in a humidified atmosphere of 5% CO_2. To clarify the effects of isorhamnetin on atrial myocytes, HL-1 cells were seeded in a 6-well plate precoated with a solution of 0.02% (w/v) gelatin containing 5 μg/mL fibronectin. HL-1 cells were treated with AngII (1 μM) for 24 h with (AngII +isorhamnetin group) or without (AngII group) isorhamnetin. In the AngII+isorhamnetin group, cells were pretreated with isorhamnetin 1 h before AngII exposure. The cells were harvested for further analysis.

2.3 Electrophysiological studies

An *in vivo* electrophysiological study was performed as previously described (Feng et al., 2020; Xu et al., 2012; Xu et al., 2010). After the mice were anesthetized by intraperitoneal injection of ketamine (50 mg/kg) and xylazine (7.5 mg/kg), a 1.2-Fr quadripolar catheter with a 2-mm electrode distance (Unique Medical, Tokyo, Japan) was inserted into the right atrium via the cervical vein. The electrode catheter was placed at a site where the amplitude of the intra-atrial electrogram was higher than that of the intraventricular electrogram. To detect the atrial effective refractory period (AERP), a programmable stimulator (SEN-7203; Nihon Kohden, Tokyo, Japan) was used to deliver approximately twice the threshold current at a 2-ms duration. AERPs were measured at basic cycle lengths involving an eight basic stimulus drive train (S1 × 8) followed by an extra stimulus (S2) at 5-ms decrements. The AERPs were defined as the shortest S1–S2 intervals that could be captured. For AF induction, an atrial burst pacing protocol was performed via two poles on the electrode catheter using a programmable stimulator with 6V amplitude, 20 ms cycle length, 6 ms pulse duration, and 30 s stimulation time. Atrial burst pacing was consecutively performed five times in each mouse to calculate the AF induction rate. The AF induction rate was calculated by dividing the number of times AF was induced by the number of times burst pacing was performed. The AF duration was defined as the interval between the initiation and spontaneous termination of AF. Additionally, an *in vitro* electrophysiological study was performed using HL-1 cells. HL-1 cells were seeded in precoated multi-electrode array (MEA) dishes (6wellMEA200/30iR-Ti-tcr; Multi Channel Systems, Germany) using the same methods as previously mentioned for HL-1 cell culture. The HL-1 seeded MEA dish was set in a chamber controlled at 37°C and stimulated with ±7500 mV bipolar stimulation at 3 Hz 30 times using MEA2100 (MC-Rack, Multi Channel Systems). Data were captured using LabChart software. Waveforms were generated and filtered at 100 Hz to eliminate electrical noise. The action potential duration (APD) was measured from the waveform of the application. The frequency of abnormal delayed after depolarization

(DAD) was a calculation of how many channels out of all the channels showed the occurrence of DADs.

2.4 Ca^{2+} imaging of isolated atrial myocytes and HL-1 cells

The Ca^{2+} imaging of isolated mouse atrial myocytes was performed as previously described (Chen, Xu, Wu, Kranias, et al., 2018; Chen, Xu, Wu, Guo, et al., 2018). Mouse atrial myocytes were isolated from the three groups following a Langendorff-free procedure (Ackers-Johnson et al., 2016). Isolated atrial myocytes were loaded with 5 μM Fluo-4 AM (Invitrogen, Carlsbad, CA, USA) diluted in 20% Pluronic F-127 DMSO at 5 μM final concentration in Tyrode buffer (NaCl 140 mM, KCl 5 mM, HEPES 5 mM, NaH_2PO_4 2 mM, $MgCl_2$ 1 mM, $CaCl_2$ 2 mM, glucose 10 mM, pH 7.4) for 10 min. The cells were then washed with Tyrode's solution with 1.8 mM Ca^{2+}, washed again with fresh Tyrode's buffer, and maintained in the buffer during confocal Ca^{2+} imaging. Line-scan imaging (1.82 ms/line) of Ca^{2+} transients was performed using a Zeiss LSM 800 confocal microscope (Zeiss, Oberkochen, Germany). The imaging was performed by focusing on a single cell. Isoproterenol (1 μM) was added to the cells after the baseline had stabilized. Sparks were evaluated using the spark master plugin in the ImageJ software. F-F_0/F_0 was also calculated using the ImageJ software. Additionally, 3D images were obtained using ImageJ plugin software, which is an interactive 3D surface plot.

HL-1 cells were seeded in a coated glass bottom dish (24×32 mm, 0.16–0.19 mm, No.1-S; Matsunami) using the same methods as for the HL-1 cell culture. To keep the solution conditions constant, all Ca^{2+} imaging experiments were carried out by immersing the glass-bottom dish in the medium. The seeded HL-1 cells were then treated with Fluo-4 for 10 min. After washing with fresh medium, the cells were incubated for 10 min in a CO_2 incubator. The imaging was performed by focusing on a single cell. The scanning laser line was focused on the single cell and was positioned to include the nucleus. The frequency of abnormal Ca^{2+} waves was a calculation of how many cells out of all the cells showed the occurrence of abnormal Ca^{2+} waves.

2.5 Echocardiography

Echocardiography was performed using a Doppler echocardiography system (Vevo 2100; Visual Sonics, Toronto, Canada), and mice were anesthetized with 1% isoflurane inhalation during the examination. The left atrial diameter (LAD) was then calculated using B-mode images, and the other parameters were calculated at the end of systole and diastole using M-mode images.

2.6 Histological examination

The left atrium was fixed in 4% paraformaldehyde, embedded in paraffin, cut into 2 μm-thick sections, and stained with Masson's trichrome. Images were obtained using an optical microscope (BZX710; Keyence). The ratio of atrial fibrosis distribution was calculated by dividing the total area of fibrosis (defined as the amount of collagen deposition stained with aniline blue) by the total tissue area.

2.7 Western blotting assay

Western blotting was conducted using material extracted from the atrium, as previously described (Feng et al., 2020; Xu et al., 2012; Xu et al., 2010). Briefly, atrial tissue was homogenized with PRO-PREP protein extract (iNtRON Biotechnology Inc., Kyungki-Do, Korea), and 10 μg of protein was subjected to SDS-PAGE on a polyacrylamide gel (Bio-Rad Laboratory, Hercules, CA, USA). Subsequently, the proteins were transferred to the membrane using semi-dry electroblotting. The resulting membranes were incubated overnight at 4°C with the following primary antibodies: p-RyR2(Ser2814) (A010-31AP; Badrilla), ox-CaMKII (07-1387; Merck), CACNA1C (ab84814; Abcam), TRPC3 (ab51560; Abcam), TRPC6 (ab62461; Abcam), p-JNK (#4671S; Cell Signaling Technology), JNK (#9258; Cell Signaling Technology), p-ERK (#9101S; Cell Signaling Technology), ERK (#9102; Cell Signaling Technology), p-NF-κB p65 (Ser536) (#3033; Cell Signaling Technology), and GAPDH (#2118S; Cell Signaling Technology). The membranes were then incubated with the appropriate secondary antibodies, namely HRP-conjugated goat anti-rabbit IgG (ab6721; Abcam) or HRP-conjugated rabbit anti-mouse IgG (ab97046; Abcam), for 1 h at room temperature. Immunoreactions were detected using enhanced chemiluminescence (ECL Prime western blotting Detection; GE Healthcare).

2.8 Real-time polymerase chain reaction (PCR)

Total RNA (1 μg) extracted from atrial tissue using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA) was reverse transcribed into cDNA using the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, Inc., Waltham, MA, USA), as previously described (Aonuma et al., 2020; Feng et al., 2020; Xu et al., 2012; Xu et al., 2010). RT-PCR was performed on the ABI Prism 7500 FAST sequence detection system (Applied Biosystems, Foster City, CA, United States) using the PrimeTime Gene Expression Master Mix (Integrated DNA Technologies). The following primers were used: Nppb (Mm.PT.588584045.g), Col1a1 (Mm.PT.587562513), Tgfb1 (Mm.PT.5811254750), Ryr2 (Mm.PT.58.45974879), Camkiia (Mm.PT.58.30362101), and Cacna1c (Mm.PT.58.16361922). Gene expression was normalized to that of the housekeeping gene, 18S rRNA (4319413E; Thermo Fisher Scientific).

2.9 Statistical analysis

The data and statistical analysis comply with the recommendations of the British Journal of Pharmacology on experimental design and analysis in pharmacology (Curtis et al., 2018). Data and statistical analyses were performed with a group size of n [?] 5. The group size (n) is listed in the figure legends, respectively, and refer to the number of independent values used in the statistical analysis. The results are presented as the means ± SEM without outlier removal. All data were tested for normality with the Shapiro-Wilk test, and statistical comparisons between the three groups were conducted using one-way analysis of variance with a *post-hoc* test (Tukey) for comparison of groups with normal distribution of data. Differences were considered statistically significant at $P < 0.05$. All statistical analyses were performed using GraphPad Prism (version 9, GraphPad Software, San Diego, CA, USA).

2.10 Materials

Isorhamnetin was synthesized from quercetin as previously described (Fujifilm Wako Pure Chemical Corp., Tokyo, Japan) (Kato et al., 2016). AngII was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

3 RESULTS

3.1 Isorhamnetin treatment inhibited AngII-induced AF susceptibility in mice

To test the inhibitory effects of isorhamnetin on susceptibility to AF, an AF induction study was performed using a transvenous electrode catheter. After 30 s of burst pacing, AF was spontaneously induced and terminated (Fig. 1A). As a result, AngII significantly increased the AF induction rate and resulted in a longer AF duration than that of the control group. Isorhamnetin treatment reversed the increase in AF induction rate and AF duration prolonged by AngII (Fig. 1B-C). Next, AERP (basic cycle length (BCL) =150 ms) was measured for the three groups. Compared with the control group, AngII significantly decreased AERP, while isorhamnetin reversed this effect. AF induction rate: Control vs. AngII vs. AngII+isorhamnetin (4.0% vs. 34.3% vs. 20.0%); Induced AF duration: Control vs. AngII vs. AngII+isorhamnetin (0.5 ± 0.4 s vs. 20.4 ± 1.9 s vs. 8.6 ± 1.1 s); AERP: Control vs. AngII vs. AngII+isorhamnetin (101.7 ± 3.3 ms vs. 64.1 ± 2.4 ms vs. 78.3 ± 2.8 ms).

These results demonstrate that isorhamnetin restored shortened AERP and suppressed the vulnerability of AF.

3.2 Isorhamnetin suppressed AngII-induced abnormal diastolic

Ca^{2+} activity in atrial myocytes

To investigate the inhibitory effect of isorhamnetin on abnormal diastolic SR Ca^{2+} activity, a Ca^{2+}imaging study was performed using isolated atrial myocytes. Isolated atrial myocytes in the AngII group showed a significantly higher frequency of diastolic Ca^{2+} sparks in the presence of isoproterenol than in the controls. In contrast, isorhamnetin treatment reversed AngII-induced diastolic Ca^{2+} spark frequency (Fig. 2A-B). Additionally, AngII treatment increased the Ca^{2+} spark amplitude, whereas isorhamnetin treatment restored

it (Fig. 2C). These findings indicate that isorhamnetin has inhibitory effects on the vulnerability of AF by alleviating diastolic aberrant SR Ca^{2+} activities.

3.3 Isorhamnetin prevented AngII-induced abnormal diastolic intracellular Ca^{2+} loading in HL-1 cells

HL-1 cells can self-generate spontaneous potentials without electrical stimuli; therefore, spontaneous Ca^{2+} waves can be observed with the use of Fluo-4 (Fig. 3A). The Ca^{2+}amplitude (F/F_0) was significantly higher in the AngII treatment group than in the isorhamnetin treatment group (Fig. 3C). In addition, AngII shortened the time to peak and decay time. In contrast, shortened time to peak and decay time were reversed by isorhamnetin treatment (Fig. 3D-E). Furthermore, during the depolarization process, AngII increased the occurrence of spontaneous abnormal Ca^{2+} waves more frequently than in the control, while isorhamnetin treatment suppressed them (Fig. 3A, G). These cellular electrophysiology results suggest that isorhamnetin reversed AngII-induced abnormal Ca^{2+} loading and aberrant diastolic Ca^{2+} activity.

3.4 Isorhamnetin reversed AngII-induced APD prolongation in Hl-1 cells

Alterations in ionic currents can change the action potential (AP) morphology and cause abnormal depolarization (Jansen et al., 2019). Additionally, DADs are known to trigger abnormal AP (Tse, 2016). To clarify the effect of isorhamnetin on APD, AP morphology was captured using MEA in HL-1 cells. The AngII group had remarkably prolonged APD_{20}, APD_{50}, and APD_{90}. However, isorhamnetin reversed APD prolongation at all time points caused by AngII (Fig. 4G, H-J). Moreover, during the burst pacing process, DADs appeared in the AngII treatment group, and after pacing, aberrant spontaneous potentials were also observed in the AngII treatment group, whereas isorhamnetin diminished DADs (Fig. 4B, K). Taken together, isorhamnetin normalized the AP morphology and abrogated the occurrence of DADs.

3.5 Isorhamnetin restored AngII-induced atrial enlargement and related gene expression in AF model mice

Left atrial enlargement is closely related to structural remodeling. To confirm the suppressive effect of isorhamnetin on atrial enlargement, an echocardiography study was performed. LAD was calculated from the B-mode images (Fig. 5A). Isorhamnetin significantly restored the AngII-induced enlargement of the LAD (Fig. 5B). However, AngII and isorhamnetin did not cause significant changes in ejection fraction (EF) and fractional shortening (FS) compared to the control group (Table 1). In addition, atrial weight (AW) and body weight (BW) were assessed in all three groups (Fig. 5C). AngII remarkably increased AW/BW, but this effect was reversed by isorhamnetin. Moreover, the AngII-induced elevation in the expression levels of *natriuretic peptide B (Nppb*), a cardiac hypertrophic marker, was significantly reversed by isorhamnetin treatment (Fig. 5D).

3.6 Isorhamnetin reversed AngII-induced atrial fibrosis and related gene expression in AF model mice

Histological analysis was performed to confirm the inhibitory effect of isorhamnetin on atrial fibrosis. Images obtained from Masson trichome staining showed more disarray fibrosis in the AngII group (Fig. 6A). Furthermore, the quantitative ratio of the fibrotic area to the total area in the AngII group was significantly higher than that in the control group. However, isorhamnetin markedly suppressed AngII-induced atrial fibrosis (Fig. 6B). Tissue fibrosis normally involves the activation of the TGF-β pathway, which promotes collagen deposition. As a result, in the left atrium, two weeks of AngII infusion increased the gene expression of *Tγφβ* and*Col1a1* (collagen type I alpha 1) compared to the controls. However, isorhamnetin diminished the expression of these genes (Fig. 6C-D). These results demonstrate that isorhamnetin has inhibitory effects on fibrosis in the atria.

3.7 Effect of isorhamnetinon AF-related protein expression in atrial tissues

The pathogenesis of AF is a complex process involving not only Ca^{2+} channels, but also a variety of other molecules (Nattel et al., 2020). CaMKII oxidation usually contributes to diastolic SR Ca^{2+} leakage and Ca^{2+}

loading (Wang et al., 2018). Cav1.2, a subunit of the L-type voltage-gated calcium channel, is encoded by the calcium voltage-gated channel subunit alpha1 C (CACNA1C) and is involved in the formation of AP and the regulation of blood pressure. TRPC3 and TRPC6 are closely related to AF by promoting myofibroblast formation (Rose et al., 2012). In addition, JNK, ERK, and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-ϰB) activate oxidation, hypertrophy, fibrosis, and inflammation (Nattel et al., 2020). The mRNA expression level of *Camkii* was not significantly increased in the AngII group compared to the control group (Fig. 7.1A). On the other hand, the mRNA expression levels of *Cacna1c* and *Ryr2* were increased in the AngII group and decreased in the AngII +isorhamnetin group (Fig. 7.1B-C). The results of western blotting for Ca^{2+}-handling-related molecules and TRPCs are shown in Fig. 7.1D. The protein expression levels of ox-CaMKII and p-RyR2 at Ser 2814 were significantly increased in the AngII group and were significantly decreased in the AngII +isorhamnetin group (Fig. 7.1E-F). The elevated expression levels of TRPC3/6 and CACNA1C induced by AngII were also decreased in the AngII +isorhamnetin group (Fig. 7.1G-I). Finally, the results of western blotting for the structural remodeling-related signaling pathways are shown in Fig. 7.2A. The protein expression levels of p-ERK, p-JNK, and p-NF-ϰB were remarkably enhanced in the AngII group and notably diminished in the AngII+isorhamnetin groups (Fig. 7.2B-D). These results suggest that suppressing the overexpression of Ca^{2+}-handling and morphology-related molecules using isorhamnetin treatment may contribute to the prevention of AF vulnerability.

4 DISCUSSION

4.1 Major findings

The findings presented in this study demonstrate that isorhamnetin suppresses AngII-induced AF vulnerability through the inhibition of electrical and structural remodeling. Isorhamnetin alleviates abnormal diastolic intracellular Ca^{2+} activities by modulating CaMKII-mediated RyR2 and restores aberrant AP morphology via the regulation of Cav1.2 activation in reverse electrical remodeling. In addition, isorhamnetin prevents left atrial enlargement and severe fibrosis via the abrogation of TRPC-mediated MAPK and TGF-β pathways in reverse structural remodeling. To the best of our knowledge, this study is the first to investigate the potential mechanism by which natural flavonoids suppress vulnerability to AF.

4.2 Pharmacodynamics and pharmacokinetics of isorhamnetin

Isorhamnetin, a natural flavonoid, is commonly found in various plants and plant-delivered foods, such as wine, nuts, and olive oil. Quercetin, another plant flavanol, has been shown to have various beneficial health (Cogolludo et al., 2007; Heiss et al., 2010; Serban et al., 2016). According to reports on the bioavailability of quercetin, most absorbed quercetin is methylated and converted to isorhamnetin (Li et al., 2012; Zhang et al., 2010). Isorhamnetin has also been shown to have cardioprotective effects induced by doxorubicin hydrochloride, myocardial ischemia-reperfusion, aortic binding, and AngII infusion by suppressing cardiac oxidation, hypertrophy, and fibrosis (Aonuma et al., 2020; Gong et al., 2020; Sun et al., 2012; Sun et al., 2013; Xu et al., 2020). Additionally, previous reports have suggested the possible effects of isorhamnetin on Ca^{2+} channels and currents (Saponara et al., 2011; Zhu et al., 2005). Based on these findings, in the current study, we explored whether isorhamnetin could possibly inhibit AF vulnerability by improving morphological abnormalities and regulating Ca^{2+}-handling. In the present study, we found that AngII increased the AF induction rate, prolonged AF duration, and decreased AERP. However, these effects were reversed with isorhamnetin (Fig. 1B-D).

4.3 Effects of isorhamnetin on electrical remodeling

Electrical remodeling involves cellular Ca^{2+}-handling abnormalities that can cause arrhythmogenic post-depolarization and spontaneous ectopic beats while promoting the development of arrhythmogenic substrates that initiate and maintain re-entry (Nattel et al., 2021). CaMKII activation and enhanced reactive oxygen species (ROS) are widely known to contribute to cardiac arrhythmias (Anderson, 2015). Among ROS, the p47(phox)-mediated activation of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase plays a crucial role in CaMKII oxidation (Wang et al., 2018). Previous reports have demonstrated that CaMKII activation is initiated by the oxidation of methionine 281/282 under AngII infusion. Additionally, when

CaMKII is oxidized, it promotes the phosphorylation of ryanodine receptor type 2 (RyR2) serine-2814, which is a key downstream molecular target for the arrhythmogenesis of AngII (Wang et al., 2018). In short, through CaMKII oxidation, a diastolic SR Ca^{2+} leak from the RyR2 can be observed. Furthermore, it has been reported that CaMKII activation enhances the Ca^{2+} sensitivity of RyR2, which lowers the threshold for spontaneous Ca^{2+} release and predisposes the heart to DAD-induced arrhythmias (Wehrens, 2011). In addition, CaMKII activation affects AP morphology by changing various ionic currents and ionic channel activities related to calcium, sodium, and potassium (Thompson et al., 2017; Wagner et al., 2009). On the other hand, previous reports have indicated that isorhamnetin treatment prevents ROS production by suppressing the overexpression of p47 (phox), a major component of CaMKII oxidation (Romero et al., 2009; Sanchez et al., 2007). Furthermore, a previous report demonstrated that isorhamnetin could modulate Ca^{2+}channels (Cav1.2) and Ca^{2+} currents, which play an important role in AP morphology (Saponara et al., 2011). In our study, AngII induced diastolic SR Ca^{2+} sparks and CaMKII oxidation. However, isorhamnetin diminished the occurrence of diastolic SR Ca^{2+} sparks, the phosphorylation of RyR2 at serin 2814, and the oxidation of CaMKII in the atrium (Fig. 2B, Fig. 7.1E-F). In addition, although AngII promoted APD prolongation and DAD formation, isorhamnetin brought the wave morphology closer to that of the control group and diminished the occurrence of DADs (Fig. 4K). Aberrant spontaneous Ca^{2+} waves were observed more frequently in the AngII group than in the control and isorhamnetin groups (Fig. 3F). These spontaneous abnormal Ca^{2+} waves may be caused by arrhythmogenic depolarizations, such as DADs. Interestingly, isorhamnetin also normalized the elevated expression of CACNA1C, which encodes Cav1.2 (Fig. 7.1C, G). This may have contributed to the normalization of wave morphology (Fig. 4G). Collectively, these results suggest that isorhamnetin reduces abnormal diastolic intracellular Ca^{2+} activity and the occurrence of DADs by abrogating CaMKII oxidation and RyR2 phosphorylation, while isorhamnetin normalizes AP morphology by modulating Ca^{2+} channels and currents.

4.4 Effects of isorhamnetin on structural remodeling.

The findings of our study also suggest that the inhibitory effect of isorhamnetin on structural remodeling may be another factor involved in its proarrhythmic effects. JNK, a major member of the MAPK family, is activated in response to stress. It has been reported that JNK activation leads to cell proliferation, apoptosis, ROS production, cytokine production, fibrosis, and hypertrophy. Moreover, JNK promotes diastolic SR Ca^{2+}leakage from the RyR2 channel through CaMKII activation (Nattel et al., 2020). In addition, ERK is a Thr/Ser kinase that leads to cell proliferation, cell differentiation, cell cycle regulation, cell apoptosis, and tissue formation when activated. In addition, ERK activation is associated with CaMKII activation (Lu et al., 2009). Furthermore, the importance of TRPCs in the Ca^{2+}signaling mechanism of cardiomyocytes has recently been discussed (Tse, 2016). TRPCs are transmembrane non-selective cation channels that play important roles in various biological functions, including neuronal cell survival, immune cell maturation, cardiac fibrosis, and hypertrophy. TRPCs have also been associated with arrhythmic remodeling stimuli by modulating Ca^{2+} entry via cardiac fibroblast responses (Rose et al., 2012). Among these, the role of TRPC3/6 arrhythmias, including AF, has been discussed (Harada et al., 2012; Nikolova-Krstevski et al., 2017). TRPC3 regulates cardiac fibroblast function via Ca^{2+}-dependent ERK phosphorylation (Harada et al., 2012). In contrast, TRPC6 contributes to the regulation of myofibroblast formation caused by endothelin-1 via JNK signaling (Nishida et al., 2007). Although some reports have shown that isorhamnetin inhibits cardiac remodeling by suppressing the JNK and ERK pathways, there are no reports indicating the evaluation of Ca^{2+} channels and molecules related to the suppression of ERK and JNK and their upstream or downstream pathways (Sun et al., 2012; Sun et al., 2013). Our study showed that isorhamnetin reversed JNK and ERK activation and TRPC3/6 protein expression induced by AngII (Fig. 7.1H-I, Fig. 7.2B-C). These results suggest that isorhamnetin diminishes CaMKII activation and normalizes Ca^{2+} entry through Ca^{2+}channels to suppress the expression of these proteins. Moreover, similar to the results shown previously for ventricles, isorhamnetin morphologically inhibited atrial fibrosis and hypertrophy and reversed the related gene expression (Fig. 5A-D, Fig. 6A-D). Isorhamnetin also suppressed the AngII-induced upregulation of NF-$\varkappa$B, which is the most fundamental indicator of inflammation and crosstalk with MAPK (Fig. 7.2D). These results emphasize the assertion that isorhamnetin strongly diminishes AngII-induced fibrosis and the

enlargement of the atrium via normalized TRPC-mediated MAPK expression.

4.5 Potential limitations

In this study, the AngII-induced AF mouse model had limitations in its ability to evaluate arrhythmic substrates related to clinical AF. Other models, such as myocardial infarction-induced AF and burst pacing-induced AF, will need to be investigated in future studies. Furthermore, the application of this study to larger mammals should also be considered, and an applied study involving patients with AF is indispensable. Additionally, although no serious side effects of isorhamnetin have been reported, an appropriate dosage should also be considered in studies involving humans.

4.6 Implications

In the past, steroids, HMG-CoA reductase inhibitors, and angiotensin II receptor blockers (ARB) have been recognized as "upstream therapies" for AF treatment. However, these drugs have many limitations, including adverse effects and limited efficacy (Nattel et al., 2021; Sarrias & Bayes-Genis, 2018) Thus, preventive therapy for AF has not been completely established. Therefore, there is an urgent need for the discovery of effective compounds or substances with fewer side effects. Recently, flavonoids have been shown to reduce the development of AF in high-risk patients. Further detailed clinical trials and animal experiments may be accelerated in the future. To the best of our knowledge, this is the first time that a natural flavonoid has been shown to play an important role in inhibiting the development of AF by modulating both structural and electrical remodeling of the atria. Given the current skepticism regarding existing preventive drugs and the great interest in the development of novel substances, this discovery could have important therapeutic implications. Furthermore, the results of this study should facilitate and accelerate investigations of the therapeutic effects of other natural compounds on AF.

4.7 Conclusion

Our results revealed that isorhamnetin suppressed AF vulnerability by modulating CaMKII-RyR2 and TRPC-mediated MAPK pathways. Our findings further reveal the therapeutically important regulatory functions of isorhamnetin against Ca^{2+}handling in cardiology. The present study not only highlights the potential of isorhamnetin as an anti-arrhythmogenic pharmaceutical or dietary supplement, but also provides a basal evaluation system to verify the anti-arrhythmic effects of other potential substances. Further studies will be needed to investigate the therapeutic effects of isorhamnetin on AF in clinical settings.

References

Ackers-Johnson, M., Li, P. Y., Holmes, A. P., O'Brien, S. M., Pavlovic, D., & Foo, R. S. (2016, Sep 30). A Simplified, Langendorff-Free Method for Concomitant Isolation of Viable Cardiac Myocytes and Nonmyocytes From the Adult Mouse Heart. *Circ Res, 119* (8), 909-920.*https://doi.org/10.1161/CIRCRESAHA.116.309202*

Anderson, M. E. (2015, Dec). Oxidant stress promotes disease by activating CaMKII. *J Mol Cell Cardiol, 89* (Pt B), 160-167.*https://doi.org/10.1016/j.yjmcc.2015.10.014*

Aonuma, K., Ferdousi, F., Xu, D., Tominaga, K., & Isoda, H. (2020). Effects of Isorhamnetin in Human Amniotic Epithelial Stem Cells in vitro and Its Cardioprotective Effects in vivo. *Front Cell Dev Biol, 8* , 578197.*https://doi.org/10.3389/fcell.2020.578197*

Bondonno, N. P., Murray, K., Bondonno, C. P., Lewis, J. R., Croft, K. D., Kyrø, C., Gislason, G., Tjønneland, A., Scalbert, A., Cassidy, A., Piccini, J. P., Overvad, K., Hodgson, J. M., & Dalgaard, F. (2020, Dec). Flavonoid intake and its association with atrial fibrillation.*Clin Nutr, 39* (12), 3821-3828.*https://doi.org/10.1016/j.clnu.2020.04.025*

Calkins, H., Hindricks, G., Cappato, R., Kim, Y. H., Saad, E. B., Aguinaga, L., Akar, J. G., Badhwar, V., Brugada, J., Camm, J., Chen, P. S., Chen, S. A., Chung, M. K., Nielsen, J. C., Curtis, A. B., Davies, D. W., Day, J. D., d'Avila, A., de Groot, N., Di Biase, L., Duytschaever, M., Edgerton, J. R., Ellenbogen, K. A.,

Ellinor, P. T., Ernst, S., Fenelon, G., Gerstenfeld, E. P., Haines, D. E., Haissaguerre, M., Helm, R. H., Hylek, E., Jackman, W. M., Jalife, J., Kalman, J. M., Kautzner, J., Kottkamp, H., Kuck, K. H., Kumagai, K., Lee, R., Lewalter, T., Lindsay, B. D., Macle, L., Mansour, M., Marchlinski, F. E., Michaud, G. F., Nakagawa, H., Natale, A., Nattel, S., Okumura, K., Packer, D., Pokushalov, E., Reynolds, M. R., Sanders, P., Scanavacca, M., Schilling, R., Tondo, C., Tsao, H. M., Verma, A., Wilber, D. J., & Yamane, T. (2017, Oct). 2017 HRS/EHRA/ECAS/APHRS/SOLAECE expert consensus statement on catheter and surgical ablation of atrial fibrillation. *Heart Rhythm, 14* (10), e275-e444.*https://doi.org/10.1016/j.hrthm.2017.05.012*

Chen, M., Xu, D., Wu, A. Z., Kranias, E., Lin, S. F., Chen, P. S., & Chen, Z. (2018, Oct). Phospholamban regulates nuclear Ca(2+) stores and inositol 1,4,5-trisphosphate mediated nuclear Ca(2+) cycling in cardiomyocytes. *J Mol Cell Cardiol, 123* , 185-197.*https://doi.org/10.1016/j.yjmcc.2018.09.008*

Chen, M., Xu, D. Z., Wu, A. Z., Guo, S., Wan, J., Yin, D., Lin, S. F., Chen, Z., Rubart-von der Lohe, M., Everett, T. H. t., Qu, Z., Weiss, J. N., & Chen, P. S. (2018, Nov 15). Concomitant SK current activation and sodium current inhibition cause J wave syndrome. *JCI Insight, 3* (22).*https://doi.org/10.1172/jci.insight.122329*

Cogolludo, A., Frazziano, G., Briones, A. M., Cobeño, L., Moreno, L., Lodi, F., Salaices, M., Tamargo, J., & Perez-Vizcaino, F. (2007, Jan 15). The dietary flavonoid quercetin activates BKCa currents in coronary arteries via production of H2O2. Role in vasodilatation.*Cardiovasc Res, 73* (2), 424-431.*https://doi.org/10.1016/j.cardiores.2006.09.008*

Curtis, M. J., Alexander, S., Cirino, G., Docherty, J. R., George, C. H., Giembycz, M. A., Hoyer, D., Insel, P. A., Izzo, A. A., Ji, Y., MacEwan, D. J., Sobey, C. G., Stanford, S. C., Teixeira, M. M., Wonnacott, S., & Ahluwalia, A. (2018, Apr). Experimental design and analysis and their reporting II: updated and simplified guidance for authors and peer reviewers. *Br J Pharmacol, 175* (7), 987-993.*https://doi.org/10.1111/bph.14153*

Feng, D., Xu, D., Murakoshi, N., Tajiri, K., Qin, R., Yonebayashi, S., Okabe, Y., Li, S., Yuan, Z., Aonuma, K., & Ieda, M. (2020, Jun 30). Nicotinamide Phosphoribosyltransferase (Nampt)/Nicotinamide Adenine Dinucleotide (NAD) Axis Suppresses Atrial Fibrillation by Modulating the Calcium Handling Pathway. *Int J Mol Sci, 21* (13).*https://doi.org/10.3390/ijms21134655*

Ganbold, M., Owada, Y., Ozawa, Y., Shimamoto, Y., Ferdousi, F., Tominaga, K., Zheng, Y. W., Ohkohchi, N., & Isoda, H. (2019, Nov 7). Isorhamnetin Alleviates Steatosis and Fibrosis in Mice with Nonalcoholic Steatohepatitis. *Sci Rep, 9* (1), 16210.*https://doi.org/10.1038/s41598-019-52736-y*

Gong, G., Guan, Y. Y., Zhang, Z. L., Rahman, K., Wang, S. J., Zhou, S., Luan, X., & Zhang, H. (2020, Aug). Isorhamnetin: A review of pharmacological effects. *Biomed Pharmacother, 128* , 110301.*https://doi.org/10.1016/j.biopha.2020.110301*

Harada, M., Luo, X., Qi, X. Y., Tadevosyan, A., Maguy, A., Ordog, B., Ledoux, J., Kato, T., Naud, P., Voigt, N., Shi, Y., Kamiya, K., Murohara, T., Kodama, I., Tardif, J. C., Schotten, U., Van Wagoner, D. R., Dobrev, D., & Nattel, S. (2012, Oct 23). Transient receptor potential canonical-3 channel-dependent fibroblast regulation in atrial fibrillation. *Circulation, 126* (17), 2051-2064.*https://doi.org/10.1161/CIRCULATIONAHA.112.121830*

He, X., Gao, X., Peng, L., Wang, S., Zhu, Y., Ma, H., Lin, J., & Duan, D. D. (2011, Jan 21). Atrial fibrillation induces myocardial fibrosis through angiotensin II type 1 receptor-specific Arkadia-mediated downregulation of Smad7. *Circ Res, 108* (2), 164-175.*https://doi.org/10.1161/CIRCRESAHA.110.234369*

Heiss, C., Keen, C. L., & Kelm, M. (2010, Nov). Flavanols and cardiovascular disease prevention. *Eur Heart J, 31* (21), 2583-2592.*https://doi.org/10.1093/eurheartj/ehq332*

Jansen, H. J., Mackasey, M., Moghtadaei, M., Liu, Y., Kaur, J., Egom, E. E., Tuomi, J. M., Rafferty, S. A., Kirkby, A. W., & Rose, R. A. (2019, Jan). NPR-C (Natriuretic Peptide Receptor-C) Modulates the Progression of Angiotensin II-Mediated Atrial Fibrillation and Atrial Remodeling in Mice. *Circ Arrhythm Electrophysiol, 12* (1), e006863.*https://doi.org/10.1161/CIRCEP.118.006863*

Kato, K., Ninomiya, M., Tanaka, K., & Koketsu, M. (2016, Jul 22). Effects of Functional Groups and Sugar Composition of Quercetin Derivatives on Their Radical Scavenging Properties. *J Nat Prod, 79* (7), 1808-1814.*https://doi.org/10.1021/acs.jnatprod.6b00274*

Li, G., Zeng, X., Xie, Y., Cai, Z., Moore, J. C., Yuan, X., Cheng, Z., & Ji, G. (2012, Jan). Pharmacokinetic properties of isorhamnetin, kaempferol and quercetin after oral gavage of total flavones of Hippophae rhamnoides L. in rats using a UPLC-MS method.*Fitoterapia, 83* (1), 182-191.*https://doi.org/10.1016/j.fitote.2011.10.012*

Lu, Y. M., Shioda, N., Han, F., Kamata, A., Shirasaki, Y., Qin, Z. H., & Fukunaga, K. (2009, Spring). DY-9760e inhibits endothelin-1-induced cardiomyocyte hypertrophy through inhibition of CaMKII and ERK activities. *Cardiovasc Ther, 27* (1), 17-27.*https://doi.org/10.1111/j.1755-5922.2008.00068.x*

Nattel, S., & Harada, M. (2014, Jun 10). Atrial remodeling and atrial fibrillation: recent advances and translational perspectives. *J Am Coll Cardiol, 63* (22), 2335-2345.*https://doi.org/10.1016/j.jacc.2014.02.555*

Nattel, S., Heijman, J., Zhou, L., & Dobrev, D. (2020, Jun 19). Molecular Basis of Atrial Fibrillation Pathophysiology and Therapy: A Translational Perspective. *Circ Res, 127* (1), 51-72.*https://doi.org/10.1161/CIRCRESAHA.120.316363*

Nattel, S., Sager, P. T., Hüser, J., Heijman, J., & Dobrev, D. (2021). Why translation from basic discoveries to clinical applications is so difficult for atrial fibrillation and possible approaches to improving it. *Cardiovascular Research, 117* (7), 1616-1631.*https://doi.org/10.1093/cvr/cvab093*

Nikolova-Krstevski, V., Wagner, S., Yu, Z. Y., Cox, C. D., Cvetkovska, J., Hill, A. P., Huttner, I. G., Benson, V., Werdich, A. A., MacRae, C., Feneley, M. P., Friedrich, O., Martinac, B., & Fatkin, D. (2017, Oct). Endocardial TRPC-6 Channels Act as Atrial Mechanosensors and Load-Dependent Modulators of Endocardial/Myocardial Cross-Talk.*JACC Basic Transl Sci, 2* (5), 575-590.*https://doi.org/10.1016/j.jacbts.2017.05.006*

Nishida, M., Onohara, N., Sato, Y., Suda, R., Ogushi, M., Tanabe, S., Inoue, R., Mori, Y., & Kurose, H. (2007, Aug 10). Galpha12/13-mediated up-regulation of TRPC6 negatively regulates endothelin-1-induced cardiac myofibroblast formation and collagen synthesis through nuclear factor of activated T cells activation. *J Biol Chem, 282* (32), 23117-23128.*https://doi.org/10.1074/jbc.M611780200*

Romero, M., Jimenez, R., Sanchez, M., Lopez-Sepulveda, R., Zarzuelo, M. J., O'Valle, F., Zarzuelo, A., Perez-Vizcaino, F., & Duarte, J. (2009, Jan). Quercetin inhibits vascular superoxide production induced by endothelin-1: Role of NADPH oxidase, uncoupled eNOS and PKC.*Atherosclerosis, 202* (1), 58-67.*https://doi.org/10.1016/j.atherosclerosis.2008.03.007*

Rose, R. A., Belke, D. D., Maleckar, M. M., & Giles, W. R. (2012, Oct 23). Ca(2+) entry through TRP-C channels regulates fibroblast biology in chronic atrial fibrillation. *Circulation, 126* (17), 2039-2041.*https://doi.org/10.1161/CIRCULATIONAHA.112.138065*

Sanchez, M., Lodi, F., Vera, R., Villar, I. C., Cogolludo, A., Jimenez, R., Moreno, L., Romero, M., Tamargo, J., Perez-Vizcaino, F., & Duarte, J. (2007, Apr). Quercetin and isorhamnetin prevent endothelial dysfunction, superoxide production, and overexpression of p47phox induced by angiotensin II in rat aorta. *J Nutr, 137* (4), 910-915.*https://doi.org/10.1093/jn/137.4.910*

Saponara, S., Carosati, E., Mugnai, P., Sgaragli, G., & Fusi, F. (2011, Nov). The flavonoid scaffold as a template for the design of modulators of the vascular Ca(v) 1.2 channels. *Br J Pharmacol, 164* (6), 1684-1697.*https://doi.org/10.1111/j.1476-5381.2011.01476.x*

Sarrias, A., & Bayes-Genis, A. (2018, Aug 7). Is Sacubitril/Valsartan (Also) an Antiarrhythmic Drug? *Circulation, 138* (6), 551-553.*https://doi.org/10.1161/CIRCULATIONAHA.118.034755*

Serban, M. C., Sahebkar, A., Zanchetti, A., Mikhailidis, D. P., Howard, G., Antal, D., Andrica, F., Ahmed, A., Aronow, W. S., Muntner, P., Lip, G. Y., Graham, I., Wong, N., Rysz, J., & Banach, M. (2016, Jul 12).

Effects of Quercetin on Blood Pressure: A Systematic Review and Meta-Analysis of Randomized Controlled Trials. *J Am Heart Assoc, 5* (7).*https://doi.org/10.1161/jaha.115.002713*

Sun, B., Sun, G. B., Xiao, J., Chen, R. C., Wang, X., Wu, Y., Cao, L., Yang, Z. H., & Sun, X. B. (2012, Feb). Isorhamnetin inhibits H(2)O(2)-induced activation of the intrinsic apoptotic pathway in H9c2 cardiomyocytes through scavenging reactive oxygen species and ERK inactivation. *J Cell Biochem, 113* (2), 473-485.*https://doi.org/10.1002/jcb.23371*

Sun, J., Sun, G., Meng, X., Wang, H., Luo, Y., Qin, M., Ma, B., Wang, M., Cai, D., Guo, P., & Sun, X. (2013). Isorhamnetin protects against doxorubicin-induced cardiotoxicity in vivo and in vitro. *PLoS One, 8* (5), e64526.*https://doi.org/10.1371/journal.pone.0064526*

Thompson, C. H., Hawkins, N. A., Kearney, J. A., & George, A. L., Jr. (2017, Feb 14). CaMKII modulates sodium current in neurons from epileptic Scn2a mutant mice. *Proc Natl Acad Sci U S A, 114* (7), 1696-1701.*https://doi.org/10.1073/pnas.1615774114*

Tse, G. (2016, Apr). Mechanisms of cardiac arrhythmias. *J Arrhythm, 32* (2), 75-81.*https://doi.org/10.1016/j.joa.2015.11.003*

Wagner, S., Hacker, E., Grandi, E., Weber, S. L., Dybkova, N., Sossalla, S., Sowa, T., Fabritz, L., Kirchhof, P., Bers, D. M., & Maier, L. S. (2009, Jun). Ca/calmodulin kinase II differentially modulates potassium currents. *Circ Arrhythm Electrophysiol, 2* (3), 285-294.*https://doi.org/10.1161/circep.108.842799*

Wang, Q., Quick, A. P., Cao, S., Reynolds, J., Chiang, D. Y., Beavers, D., Li, N., Wang, G., Rodney, G. G., Anderson, M. E., & Wehrens, X. H. T. (2018, Apr). Oxidized CaMKII (Ca(2+)/Calmodulin-Dependent Protein Kinase II) Is Essential for Ventricular Arrhythmia in a Mouse Model of Duchenne Muscular Dystrophy. *Circ Arrhythm Electrophysiol, 11* (4), e005682.*https://doi.org/10.1161/CIRCEP.117.005682*

Wehrens, X. H. (2011, Feb). CaMKII regulation of the cardiac ryanodine receptor and sarcoplasmic reticulum calcium release. *Heart Rhythm, 8* (2), 323-325.*https://doi.org/10.1016/j.hrthm.2010.09.079*

Williams, B. A., Chamberlain, A. M., Blankenship, J. C., Hylek, E. M., & Voyce, S. (2020, Aug 3). Trends in Atrial Fibrillation Incidence Rates Within an Integrated Health Care Delivery System, 2006 to 2018.*JAMA Netw Open, 3* (8), e2014874.*https://doi.org/10.1001/jamanetworkopen.2020.14874*

Xu, D., Murakoshi, N., Igarashi, M., Hirayama, A., Ito, Y., Seo, Y., Tada, H., & Aonuma, K. (2012, Feb). PPAR-gamma activator pioglitazone prevents age-related atrial fibrillation susceptibility by improving antioxidant capacity and reducing apoptosis in a rat model. *J Cardiovasc Electrophysiol, 23* (2), 209-217.*https://doi.org/10.1111/j.1540-8167.2011.02186.x*

Xu, D., Murakoshi, N., Tada, H., Igarashi, M., Sekiguchi, Y., & Aonuma, K. (2010, Jan). Age-related increase in atrial fibrillation induced by transvenous catheter-based atrial burst pacing: an in vivo rat model of inducible atrial fibrillation. *J Cardiovasc Electrophysiol, 21* (1), 88-93.*https://doi.org/10.1111/j.1540-8167.2009.01591.x*

Xu, Y., Tang, C., Tan, S., Duan, J., Tian, H., & Yang, Y. (2020, Jun). Cardioprotective effect of isorhamnetin against myocardial ischemia reperfusion (I/R) injury in isolated rat heart through attenuation of apoptosis. *J Cell Mol Med, 24* (11), 6253-6262.*https://doi.org/10.1111/jcmm.15267*

Zhang, L., Angst, E., Park, J. L., Moro, A., Dawson, D. W., Reber, H. A., Eibl, G., Hines, O. J., Go, V. L., & Lu, Q. Y. (2010, Jun 23). Quercetin aglycone is bioavailable in murine pancreas and pancreatic xenografts. *J Agric Food Chem, 58* (12), 7252-7257.*https://doi.org/10.1021/jf101192k*

Zhu, F., Huang, B., Hu, C. Y., Jiang, Q. Y., Lu, Z. G., Lu, M., Wang, M. H., Gong, M., Qiao, C. P., Chen, W., & Huang, P. H. (2005, Dec). Effects of total flavonoids of Hippophae rhamnoides L. on intracellular free calcium in cultured vascular smooth muscle cells of spontaneously hypertensive rats and Wistar-Kyoto rats. *Chin J Integr Med, 11* (4), 287-292.*https://doi.org/10.1007/bf02835791*

Table 1.

Echocardiographic parameters

$^{*}P < 0.05$ control vs. AngII by one-way ANOVA with*post-hoc* test (Tukey);$^{\#}P < 0.05$ AngII vs. AngII+isorhamnetin by one-way ANOVA with *post-hoc* test (Tukey) (control, n = 5; AngII, n = 5; AngII+isorhamnetin, n = 5).

LAD: Left atrial dimension. LVID, left ventricular internal dimension; EF, ejection fraction; FS, fractional shortening; LV Vol, LV volume; quantification of LAD calculated from B-mode images. Quantification of other parameters calculated from M-mode images at the end of systole and diastole.

Figure legends

Figure 1. Isorhamnetin suppressed AngII-induced AF vulnerability in mice.

(A) Representative intracardiac and surface electrogram traces of burst pacing-induced AF. (B) Summary of data for AF induction rate (control, n = 10; AngII, n = 7; AngII+isorhamnetin, n = 9). (C) Duration of pacing-induced AF. (D) AERP (BCL = 150 msec) (control, n = 6 AngII, n = 6; AngII+isorhamnetin, n = 6). $^{**}P < 0.01$ control vs. AngII by one-way ANOVA with *post-hoc* test (Tukey);$^{\#\#}P < 0.01$ AngII vs. AngII+isorhamnetin; $^{\#}P < 0.05$ AngII vs. AngII+isorhamnetin by one-way ANOVA with *post-hoc* test (Tukey).

Figure 2. Isorhamnetin inhibited AngII-induced diastolic abnormal Ca^{2+} activities in atrial myocyte.

(A) Representative confocal line scan images showing Ca^{2+} sparks in atrial myocytes isolated from three groups under stimulation with Isoproterenol (1 μM). (B) Quantification of Ca^{2+} spark frequency (CaSpF) analyzed using the spark master plugin on ImageJ software. (C) Quantification of Ca^{2+} amplitude (F-F_0/F_0) analyzed using ImageJ software (control, n = 23 cells from 5 mice; AngII, n = 29 cells from 5 mice; AngII+isorhamnetin, n = 21 cells from 5 mice). $^{**}P < 0.01$ control vs. AngII by one-way ANOVA with *post-hoc* test (Tukey); $^{\#\#}P < 0.01$ AngII vs. AngII+isorhamnetin by one-way ANOVA with *post-hoc* test (Tukey).

Figure 3. Isorhamnetin alleviated AngII-induced abnormal diastolic Ca^{2+} loading in HL-1 cells.

(A) Representative confocal line scan images showing Ca^{2+} waves in HL-1 cells from three groups. Red arrows show abnormal Ca^{2+} wave. (B) Representative fluorescence intensity. Quantification of Ca^{2+}diastolic [Ca^{2+}]: (C) amplitude; (D) time to peak; (E) decay time; (F) wave frequency in 30 s. (G) The frequency of abnormal Ca^{2+} wave from three groups (control, n = 6; AngII, n = 7; AngII+isorhamnetin, n = 6). $^{**}P < 0.01$ control vs. AngII by one-way ANOVA with *post-hoc* test (Tukey);$^{\#\#}P < 0.01$ AngII vs. AngII+isorhamnetin; $^{\#}P < 0.05$ AngII vs. AngII+isorhamnetin by one-way ANOVA with *post-hoc* test (Tukey).

Figure 4. Isorhamnetin restored AngII-induced prolonged APD in Hl-1 cells.

(A-C) Representative images before and after burst pacing with HL-1 cells spontaneous potentials. (D-F) Representative images of single AP. Red arrows show abnormal APs. (G) Representative images of merged three consecutive action potentials from three groups. Quantification of APD: (H) APD 20; (I) APD 50; (J) APD 90 from three groups. (K) Frequency of DADs (control, n = 15 from 5 channels; AngII, n = 15 from 5 channels; AngII+isorhamnetin, n = 15 from 5 channels). $^{**}P < 0.01$ control vs. AngII by one-way ANOVA with *post-hoc* test (Tukey);$^{\#\#}P < 0.01$ AngII vs. AngII+isorhamnetin by one-way ANOVA with *post-hoc* test (Tukey).

Figure 5. Effect of isorhamnetin obtained by echocardiographic findings.

(A) Representative echocardiographic images from B-Mode. (B) Quantification of LAD (control, n = 5; AngII, n = 5; AngII+isorhamnetin, n = 5). (C) Quantification of AW/BW (control, n = 6; AngII, n =

6; AngII+isorhamnetin, n = 6). (D) Quantification of levels of *Nppb* . qRT-PCR analysis of mRNA levels normalized with 18 s (control, n = 6; AngII, n = 6; AngII+isorhamnetin, n = 6). $^{**}P < 0.01$ control vs. AngII; $^{*}P < 0.05$ control vs. AngII by one-way ANOVA with *post-hoc* test (Tukey);$^{\#\#}P < 0.01$ AngII vs. AngII+isorhamnetin; $^{\#}P < 0.05$ AngII vs. AngII+isorhamnetin by one-way ANOVA with *post-hoc* test (Tukey).

Figure 6. Effect of isorhamnetin obtained by immunohistological findings.

(A) Representative Masson's trichrome images from three groups in the atria. (B) Quantification of fibrotic area/total area (control, n = 5; AngII, n = 5; AngII+isorhamnetin, n = 5). (C-D) Quantification of levels of *Col1a1* , *Tgfb1* . qRT-PCR analysis of mRNA levels normalized with 18 s (control, n = 6; AngII, n = 6; AngII+isorhamnetin, n = 6). $^{**}P < 0.01$ control vs. AngII; $^{*}P< 0.05$ control vs. AngII by one-way ANOVA with *post-hoc*test (Tukey); $^{\#\#}P < 0.01$ AngII vs. AngII+isorhamnetin; $^{\#}P < 0.05$ AngII vs. AngII+isorhamnetin by one-way ANOVA with *post-hoc* test (Tukey).

Figure 7. Effect of isorhamnetin based on western botting and RT-PCR findings.

(7.1A-C) Quantification of the levels of *Camkii* , *Ryr2* , and*Cacna1c* . qRT-PCR analysis of mRNA levels normalized to 18 s (control, n = 6; AngII, n = 6; AngII+isorhamnetin, n = 6). (7.1D) Representative images obtained from western blotting using atrial tissue, and western blot analysis with ox-CAMKII, p-RyR2 (Ser2814), TRPC3, TRPC6, and CACNA1C. (7.1E-I) Quantification of intensity analyzed using western blot images (control, n = 6; Ang II, n = 6; AngII+isorhamnetin, n = 6). $^{**}P < 0.01$ control vs. AngII; $^{*}P < 0.05$ control vs. AngII by one-way ANOVA with *post-hoc* test (Tukey); $^{\#\#}P< 0.01$ AngII vs. AngII+isorhamnetin;$^{\#}P < 0.05$ AngII vs. AngII+isorhamnetin by one-way ANOVA with *post-hoc* test (Tukey). (7.2A) Representative images obtained by western blotting of atrial tissue. Western blot analysis with p-JNK, JNK, p-ERK, ERK, p-NF-ϰB, and GAPDH. (7.2B-D) Quantification of intensity analyzed by western blot images (control, n = 6; AngII, n = 6; AngII+isorhamnetin, n = 6). $^{**}P < 0.01$ control vs. AngII by one-way ANOVA with*post-hoc* test (Tukey). $^{\#\#}P < 0.01$ AngII vs. AngII+isorhamnetin; $^{\#}P<$ 0.05 AngII vs. AngII+isorhamnetin by one-way ANOVA with*post-hoc* test (Tukey).

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