Up-regulation of myocardial connexin-43 in spontaneously hypertensive rats fed red palm oil is most likely implicated in its anti-arrhythmic effects

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Abstract: The purpose of this study was to test our hypothesis that red palm oil (RPO) intake may affect abnormalities of myocardial connexin-43 (Cx43) and protein kinase Cε (PKCε) signaling, and consequently the propensity of the spontaneously hypertensive rat heart (SHR) heart to arrhythmias. SHR and Wistar–Kyoto (WKY) rats fed a standard rat chow plus red palm oil (200 μL/day) for 5 weeks were compared with untreated rats. Cytosolic but not particulate PKCε expression as well as Cx43-mRNA, total Cx43 proteins, and its phoshorylated forms were increased, and disordered localization of Cx43 was attenuated in the left ventricle of RPO-fed SHR compared with untreated rats. These alterations were associated with suppression of early post-ischemic-reperfusion-related ventricular tachycardia and electrically inducible ventricular fibrillation. However, the treatment dose of RPO caused down-regulation of myocardial Cx43, but did not alter its cell membrane distribution or overall PKCε expression in WKY rats. It was, however, associated with poor arrhythmia protection, suggesting overdosing. Results indicate that SHR benefit from RPO intake, particularly because of its apparent anti-arrhythmic effects. This protection can be, in part, attributed to the preservation of cell-to-cell communication via up-regulation of myocardial Cx43, but not with PKCε activation.

Key words: heart, connexin-43, PKCε, arrhythmias, SHR, red palm oil.

Résumé : Le but de cette étude était de tester notre hypothèse à l'effet que l'ingestion d'huile de palme rouge (HPR) peut agir sur les anomalies de la connexine-43 (Cx43) du myocarde et la signalisation de la protéine kinase Cε (PKCε), et conséquemment agir sur la propension des cœurs de rats spontanément hypertendus (SHR) à l'arythmie. Des rats SHR et Wistar–Kyoto (WKY) nourris avec une moulée standard contenant de l'huile de palme rouge (200 μL/jour) ont été comparés à des rats non traités. Lexpression de la PKC cytosolique mais pas la PKC particulaire, de même que lexpression de lARNm de la Cx43, de la Cx43 totale et de ses formes phosphorylées étaient accrues, et la localisation désordonnée de la Cx43 était amoindrie dans le ventricule gauche des rats SHR nourris à l'HPR comparativement aux rats non traités. Ces modifications étaient associées à la suppression de la tachycardie ventriculaire liée à la reperfusion post-ischémique précoce et de la fibrillation ventriculaire inductible électriquement. Cependant, la dose de traitement d'HPR régulait à la baisse la Cx43 du myocarde mais n'affectait pas la distribution membranaire de même que l'expression globale de la PKC chez les rats WKY. Ce phénomène était associé à une faible protection contre l'arythmie, suggérant une surdose. Les résultats indiquent que les rats

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SHR peuvent bénéficier de l'ingestion d'HPR, à cause notamment de ses effets anti-arythmiques. Cette protection peut être attribuée en partie à la préservation de la communication cellule–cellule par l'intermédiaire d'une régulation à la hausse de la Cx43 du myocarde mais non à l'activation de la PKC.

Mots-clés : cœur, connexine-43, PKC, arythmie, SHR, huile de palme rouge.

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Introduction

The link between dietary fats and cardiovascular disease has initiated a growing interest in dietary red palm oil (RPO) research. In spite of its high saturated fatty acid content (50%), RPO intake has not been found to promote vascular disease. On the contrary, the benefits of RPO to health includes reduction in the risk of arterial thrombosis and (or) atherosclerosis, platelet aggregation, a reduction in blood pressure (Edem 2002), inhibition of endogenous cholesterol biosynthesis, and a reduction in oxidative stress (Das et al. 2008). Oxidative stress and the severity or progression of disease has stimulated further interest in the potential role of RPO (a cocktail of natural antioxidants) to improve redox status. The latter is probably due to the ratio of saturated (50%) to unsaturated fatty acids (40% mono- and 10% poly-unsaturated) and high concentration of antioxidants such as pro-vitamin E, namely tocotrienols, tocopherols, coenzyme Q10, and lycopene (Edem 2002; Das et al. 2008; van Rooyen et al. 2008).

Recent experimental studies demonstrated that dietary RPO supplementation offers protection against ischemia–reperfusion injury by improved cardiac output recovery (Esterhuyse et al. 2005, 2006; Kruger et al. 2007). Evidence strongly suggests that the phosphoinositide 3-kinase (PI3-K), MAPKs, NO-cGMP, and pro-survival PKB–Akt signaling pathway may be involved in (Engelbrecht et al. 2006, 2009; Bester et al. 2010; Szucs et al. 2011; van Rooyen et al. 2008). The latter mechanism has also been implicated in tocotrienol-rich palm oil induced improvement of post-ischemic ventricular function and reduction of myocardial infarct size (Das et al. 2008). Nevertheless, the other cellular and molecular mechanisms that might be involved in RPO-related cardioprotection, such as PKCε signaling that play a pivotal role in ischemia (Hund et al. 2007) and diseased heart (Lin et al. 2008) protection are still not elucidated. However, PKCε mRNA has been shown to be increased in SHR (McCrossan et al. 2004) and pharmacological inhibition of PKCε attenuated cardiac fibrosis and dysfunction in hypertensive Dahl rats (Inagaki et al. 2008). Currently no data exists indicating that RPO supplementation will affect the propensity of the heart to malignant arrhythmias in humans or experimental animals.

Following our previous studies demonstrating the protection of the heart from life-threatening ventricular fibrillation (VF) by omega-3 fatty acids (FA) in hereditary hypertriglyceridemic (Bacova et al. 2010) and spontaneously hypertensive rats (SHR) (Mitasíková et al. 2009), the question arose as to whether RPO supplementation may offer similar anti-arrhythmic protection. Moreover, we have shown that myocardial intercellular connexin-43 (Cx43) channels are most likely involved in the anti-arrhythmic mechanisms of omega-3 FA (Mitasíková et al. 2009; Bacova et al. 2010; Radošinská et al. 2011). Cardiac Cx43 channels at the gap junctions play an important role in synchronizing myocardium, allowing electrical and molecular signal propagation amongst cardiomyocytes (Dhein 1998). In turn, impaired cell-to-cell communication due to alterations in Cx43 distribution and (or) expression promotes development of malignant arrhythmias and contractile dysfunction, as shown by our and by other studies (Liu and Gutstein 2002; Tribulová et al. 2002, 2008, 2009; Salameh and Dhein 2005; Lin et al. 2008). The purpose of this study was to examine whether RPO supplementation will affect Cx43 and PKCε signaling, which has been shown to be altered in arrhythmia-prone SHR, and the consequent susceptibility of hypertensive rat heart to arrhythmias.

Material and methods

Animal monitoring and sample collection

All animal experiments were performed in accordance with the rules issued by the State Veterinary Administration of the Slovak Republic, legislation No. 289/2003, and they conform to the *European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes* (Council of Europe No. 123, Strasbourg 1985) and to the *Guide for the Care and Use of Laboratory Animals* (ILAR 1996). Male, 3-month-old spontaneously hypertensive rats (SHR) ($n = 13$) and nonhypertensive Wistar–Kyoto rats (WKY) ($n = 13$) fed a standard rat chow plus RPO (200 μL/day) for 5 weeks were compared with untreated controls ($n = 26$). The composition of RPO (Carotino Sdn Bhd, Johor Bahru, Malaysia) was as follows (*w*/*w*): total saturated fatty acids (SFA), 51%; total monounsaturated fatty acids (MUFA), 38%; and total polyunsaturated fatty acids (PUFA), 11%. The other components included (*w*/*w*) stearic acid, 5%; palmitic acid, 44%; oleic acid, 38%; linoleic acid, 11%; squaline, 1.1%; vitamin E, 560–1000 ppm; carotenoids, 500 ppm; coenzyme Q10, 0.4 mg/L.

Systolic blood pressure (BP) was measured non-invasively by tail-cuff plethysmography using the Statham pressure transducer P23XL (Hugo Sachs, Germany) at the beginning and end of the experiment; body mass (BW) was also monitored. Fasting blood glucose (BG), triglycerides (TG), and cholesterol (CH) were registered at the end of experiment using a commercial kit from Biolab Diagnostics. The hearts were rapidly excised from the anaesthetized rats and immediately placed into ice-cold saline, wherein whole heart mass and left ventricular mass were measured. Left ventricular tissue was used for real-time polymerase chain reaction (PCR) and immunoblotting to determine Cx43 mRNA and protein expressions as well as PKCε expression. Left ventricular cryostat sections were used to detect in-situ Cx43 distribution. Isolated Langendorff-perfused rat heart was used for examination of post-ischemic reperfusion-induced arrhythmias, electrically inducible ventricular fibrillation (VF), and heart function.

Real-time PCR for Cx43-mRNA assay

RNA isolation from heart tissue was performed using TRI Reagent® (Sigma–Aldrich, USA). RNA concentration was determined using NanoDrop spectrophotometer ND1000 (NanoDrop Technologies Inc, USA). Reverse transcription (RT) was realized using RevertAid™ H Minus First Strand cDNA Synthesis Kit (Fermentas), using 1.2–1.5 μg of total RNA and random-hexamer primer. Obtained single-chain DNA was used for real-time polymerase chain reaction (PCR). Amplification was performed in 10 μL of SYBR Green PCR Master Mix containing 30 pmol/L of each primer. For amplification of *GJP43* and *β-actin* (housekeeping gene) gene fragments, the following primers were used: sense, 5′-TCC TTG GTG TCT CTC GCT TT-3′; *GJP43* antisense, 5′-GAG CAG CCA TTG AAG TAG GC-3′; sense, 5′-TCA TCA CTA TCG GCA ATG AGC-3′; antisense, 5′-GGC CAG GAT AGA GCC ACC A-3′, respectively. Sample volume was adjusted to 20 μL with deionized water. Amplification was performed using the 7500 Fast Real-Time PCR system. The amplification program consisted of an initial AmpliTaq Gold® DNA polymerase activation step at 95 °C for 10 min and the following for 50 cycles: denaturation (95 °C for 15 s), annealing and elongation (56 °C for 60 s). For control of specificity, a dissociation stage was included: sequential increase of temperature from 56 °C to 99 °C, with registration of the drop in the double-stranded DNA–SYBR Green complex fluorescence strength. We performed calculations using the 7500 Fast System SDS 1.4 software provided. The CT (cycle threshold) is defined as the number of cycles required for the fluorescence signal to exceed the detection threshold. We calculated the expression of the target gene relative to the housekeeping gene as the difference between the threshold values of the 2 genes.

Western blot analysis of Cx43 and PKCε expression and subcellular distribution

For Cx43 and overall PKCε analysis, frozen tissue from the left ventricle was powdered and solubilized in SB20 solution (20% SDS, 10 mmol/L EDTA,100 mmol/L Tris, pH 6.8) by sonicator (UP 100H; Hielscher, Germany). To differentiate PKCε expression in the cytosolic and membrane-rich fraction, the left ventricular tissue was put in ice-cold buffer containing (in millimoles per litre): 50 Tris–HCl, 250 sucrose, 1.0 ethylene glycol tetraacetic acid (EGTA), 1.0 dithiothreitol, 1.0 phenylmethylsulfonyl fluoride, and 0.5 sodium orthovanadate (pH 7.4), and homogenized. The homogenates were centrifuged at 800*g* for 5 min at 4 °C, and the supernatants after this centrifugation were centrifuged again at 16 100*g* for 30 min. The supernatants after this second centrifugation represented the soluble fraction. After second centrifugation, the pellets were resuspended in a buffer containing 0.2% Triton X-100 and centrifuged at 4500*g* for 5 min. The supernatant from this centrifugation represented the particulate fraction. An equal amount of total protein in each sample was separated in 10% SDS–PAGE and transferred electrophoretically to a nitrocellulose membrane. For Cx43 determination, the membrane was incubated with primary rabbit monoclonal antibodies (Anti-Connexin 43 C 6219; Sigma–Aldrich, diluted 1:2000) to recognize the phosphorylated (P-Cx43) and un-phosphorylated (P_0-Cx43) form of Cx43. The membrane was also incubated with Zymed No. 13-8300 (diluted 1:200) mouse monoclonal antibody to recognize un-phosphorylated Cx43 only, as well as PKCε determination with primary rabbit polyclonal antibody (C-15, sc-214, Santa Cruz Biotechnology, Inc., diluted 1:1000) overnight at 4 °C, followed by further incubation for 1 h at room temperature with a secondary donkey antibody (peroxidase-labeled anti-rabbit and anti-mouse imunoglobulin, 1:2000, for Cx43 and PKCε; Amersham Biosciences). Bound antibodies were detected by the ECL method. The optical density of individual bands was analyzed by PCBAS 2.08e software and normalized to GAPDH or actin as an internal control.

In-situ immunolabeling of Cx43

Ventricular 10 μm thick cryostat sections were used for in-situ immunodetection of Cx43 using mouse monoclonal antiCx43 antibody (Chemicon International, Inc., USA) and secondary fluorescein isothiocyanate (FITC) conjugated goat anti-mouse antibody, as described previously (Bacova et al. 2010). Immunostained sections were examined in fluorescence microscope Axiostar (Car Zeiss Jena, Germany) and images were stored digitally for subsequent quantitative image analysis (Soft Imaging System, GmbH, Germany) as previously described (Bacova et al. 2010). Twenty randomly selected test areas were investigated per heart. From each test field, a histogram of Cx43 fluorescence intensity was obtained and used for calculation of the Cx43 signal. The area of positive Cx43 labeling was defined as the number of pixels with Cx43 signal intensity exceeding threshold of 30 on the 0–255 gray scale. Thereafter, the total number of Cx43 positive pixels was expressed as integral optical density (IOD) per area. The later parameter expressed in arbitrary units was compared between groups.

Experimental protocol of isolated Langendorff-perfused heart

The hearts of 5 rats from each group were perfused via canulated aorta in Langendorff mode with oxygenated Krebs–Henseleit solution at constant pressure of 80 mm Hg (1 mm Hg = 133.322 Pa) and temperature of 37 °C. Left ventricular pressure (LVP) was measured isovolumetrically by a water-filled latex balloon, which was introduced into the left ventricle through the mitral orifice and connected to a pressure transducer. Bipolar ECG, LVP, and the coronary flow (CF) were continuously monitored during experiment for the evaluation of cardiac arrhythmias and heart function. After 20 min of equilibration, the heart was subjected to 30 min of global ischemia followed by 5 min of reperfusion to test for the incidence and duration of early reperfusion-induced arrhythmias. Thereafter the susceptibility of the heart to electrically induced and sustained (2 min) VF was examined using 1 s bursts of rectangular electrical pulses (100 pulses/s), 1 ms in duration, at a current strength 35 mA (Electrostimulator ST-3″) delivered via stimulating electrodes attached to the epicardium of the right ventricle. When sustained VF occurred, the ability of the heart to restore sinus rhythm was registered, i.e., the time from stop-perfusion-induced termination of VF until the occurrence of spontaneous sinus rhythm (as previously described by Radošinská et al. 2011*a*).

Table 1. Main characteristics of RPO-treated and untreated SHR and WKY rats

	WKYc	WKYrpo	SHRc	SHRrpo
BP (mm Hg)	123.6±11.6	117.8±12.2	184.9±20.27*	160.6±13.8#
BW (g)	404.3±27.3	417.5±47.8	322.7±20.3*	321.3±25.1
HW (g)	1.25±0.09	1.21±0.16	1.31±0.1	1.23±0.1
LVW (g)	0.765±0.070	0.789±0.073	0.848±0.056	0.810±0.064
HW/BW (mg/g)	3.11±0.3	2.9±0.29	3.66±0.19*	3.3±1.34
LVW/BW (mg/g)	2.15±0.19	1.92±0.18	2.63±0.14*	2.8±0.1
BG (mmol/L)	6.38±0.84	5.5±0.43*	5.6±1.13	4.48±0.248#
TG (mmol/L)	0.53±0.06	0.462±0.09	0.360±0.03*	0.417±0.07
CH (mmol/L)	2.39±0.27	2.20±0.13	2.19±0.11	2.18±0.1

Note: Values are the mean ± SD of 13 rats in each group. RPO, red palm oil; WKYc, control group of Wistar–Kyoto rats; WKYrpo, WKY rats fed red palm oil; SHRc, control group of spontaneously hypertensive rats; SHRrpo, SHR rats fed red palm oil; BP, blood pressure; BW, body mass; HW, heart mass; LVW, left ventricular mass; BG, blood glucose; TG, triglycerides; CH, cholesterol; 1 mm Hg = 133.322 Pa. *, $p < 0.05$ compared with WKYc; #, $p < 0.05$ compared with SHRc.

Statistical analysis

The data are expressed as the mean ± SD. One-way ANOVA followed by Duncan's post hoc test and Mann–Whitney or Fisher's exact test was used to analyze the statistically significant difference between means. Comparisons between 2 groups was performed by the 2-tailed Student' *t* test. Values were considered to differ significantly at $p < 0.05$.

Results

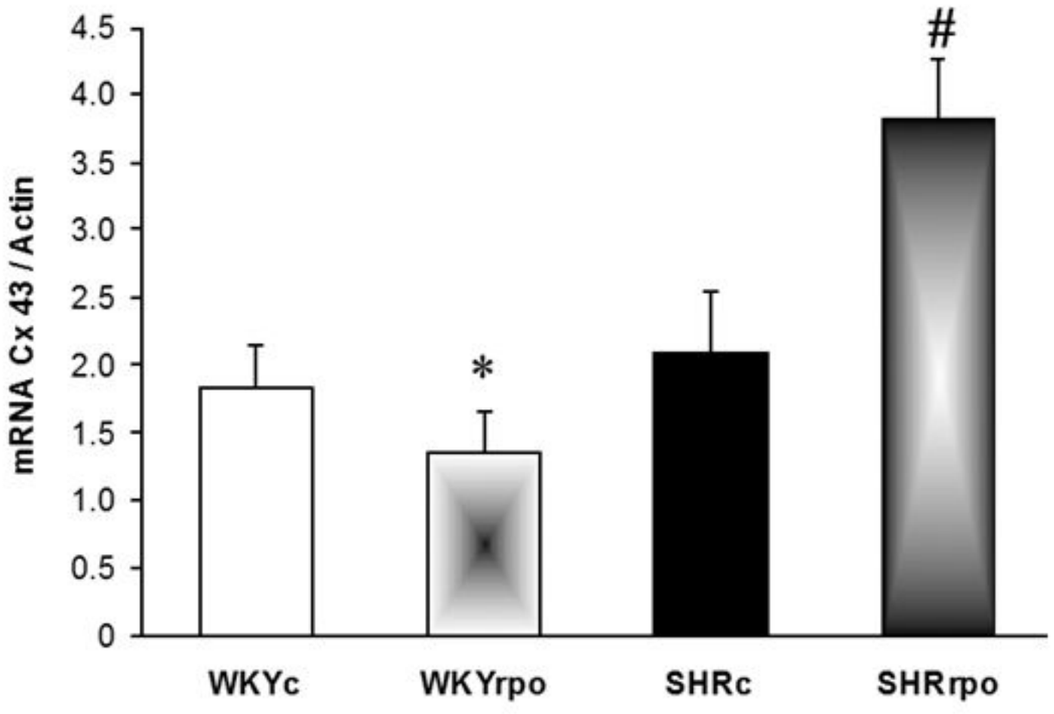
General characteristics of animals

RPO supplementation reduced BP by 13%, i.e., from 184 ± 20 to 160 ± 13 mm Hg ($p < 0.05$) in SHR, and BG in both SHR and WKY by 20% and 14%, respectively (4.48 ± 0.2 compared with 5.61 ± 1.1, and 5.5 ± 0.4 compared with 6.38 ± 0.8 mmol/L, $p < 0.05$). There were no significant differences in plasma CH and TG among the untreated and RPO-treated groups. Further, body mass, heart mass, and LVW/BW index were not affected by RPO supplementation. Data are summarized in Table 1.

Myocardial Cx43 expression and distribution

Real-time PCR showed that there is no difference in myocardial Cx43 mRNA level between WKY and SHR. However, RPO supplementation resulted in a significant increase of Cx43 mRNA in SHR, unlike the WKY rats, in which *Cx43* gene transcription was suppressed (Fig. 1). Immunoblotting revealed 3 obvious forms of myocardial Cx43, i.e., 2 functional phosphorylated (P-Cx43) and one unphosphorylated form (P_0-Cx43) in all examined rats (Fig. 2A). Compared with the WKY rats, the total Cx43 expression as well as P-Cx43 and the ratio of P-Cx43 to P_0-Cx43 were significantly reduced in untreated SHR (Figs. 2B–2D), while the level of P_0-Cx43 (detected by Zymed Cx43 antibody) was increased (Figs. 3A and 3B). However, treating WKY and SHR with an RPO-supplemented diet resulted in different response: there was a significant increase of total- and P-Cx43 in SHR heart ventricles, while these parameters were suppressed in WKY hearts (Figs. 2B and 2C) when P_0-Cx43 was enhanced (see also Figs. 3A and 3B). In ventricular tissues of WKY rats, the immunostaining of Cx43 (Fig. 4) was uniform and distributed predominantly at intercalated disk-related gap junctions (end-to-end orientation) and sporadically at lateral cell membrane gap junctions (side-to-side orientation). In contrast, an abnormal and heterogeneous distribution was detected in SHR heart ventricle that was characterized by enhanced lateral staining of Cx43-positive gap junctions and disordered intercalated disk-related staining when compared with WKY rats. This pattern of Cx43 distribution was apparently eliminated in RPO-treated SHR hearts, according to visual examination (Fig. 4), and quantitative image analysis revealed a significant increase in Cx43-positive signal compared with untreated SHR (Fig. 5). There were no changes either in cell membrane distribution or intensity of immunoreaction in RPO-treated WKY rats (Figs. 4 and 5).

Fig. 1. Connexin-43 (Cx43) mRNA expression normalized to actin in the left ventricles of untreated and RPO-treated SHR and WKY rat hearts. RPO, red palm oil; WKYc, control group (untreated) of Wistar–Kyoto rats; WKYrpo, WKY rats fed red palm oil; SHRc, control group (untreated) of spontaneously hypertensive rats; SHRrpo, SHR fed red palm oil. Results are the mean ± SD of 8 hearts per group; *, $p < 0.05$ compared with WKYc group; #, $p < 0.05$ compared with the SHRc group.



Myocardial PKCε expression

Compared with WKY rats, the expression of specific PKCε isoform was not significantly altered in the left ventricle of SHR. However, RPO supplementation resulted in a 31% increase of PKCε expression ($p < 0.05$) in the homoge-

Fig. 2. (A) Representative immunoblot showing 3 forms of connexin-43 (Cx43) and (B) densitometric quantification of total Cx43 expression, (C) its phosphorylated form, and (D) ratio of phosphorylated to unphosphorylated forms of Cx43 normalized to GAPDH in the left ventricles of untreated and RPO-fed SHR and WKY rat hearts. RPO, red palm oil; SHR, spontaneously hypertensive rats; WKY, Wistar–Kyoto rats; P0, unphosphorylated Cx43; P1 and P2, phosphorylated forms of Cx43; WKYc, control group (untreated) of WKY rats; WKYrpo, WKY rats fed red palm oil; SHRc, control group (untreated) of SHR; SHRrpo, SHR fed red palm oil. Results are the mean ± SD of 8 hearts per group; *, $p < 0.05$ compared with the WKYc group; #, $p < 0.05$ compared with SHRc.

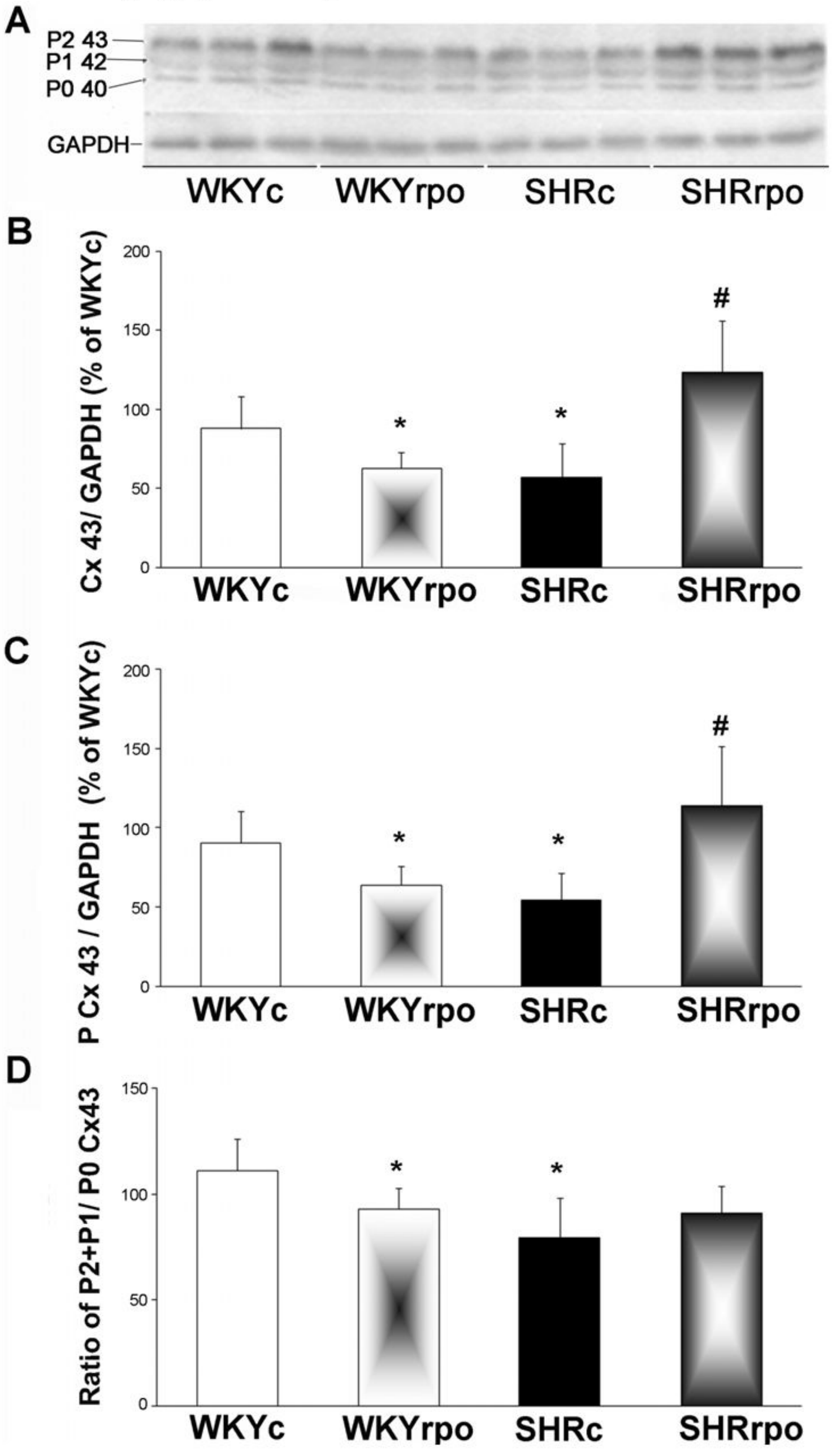


Fig. 3. (A) Representative immunoblot showing the unphosphorylated form of connexin-43 (Cx43) using Zymed No. 13-8300 antibody and (B) densitometric quantification normalized to GAPDH in the left ventricles of untreated and RPO-fed SHR and WKY rat hearts. RPO, red palm oil; SHR, spontaneously hypertensive rats; WKY, Wistar–Kyoto rats; P0, unphosphorylated form of Cx43; WKYc, control group (untreated) of WKY rats; WKYrpo WKY rats treated with RPO; SHRc, control group (untreated) of SHR; SHRrpo, SHR treated with RPO. Results are the mean ± SD of 8 hearts per group; *, $p < 0.05$ compared with WKYc; #, $p < 0.05$ compared with SHRc.

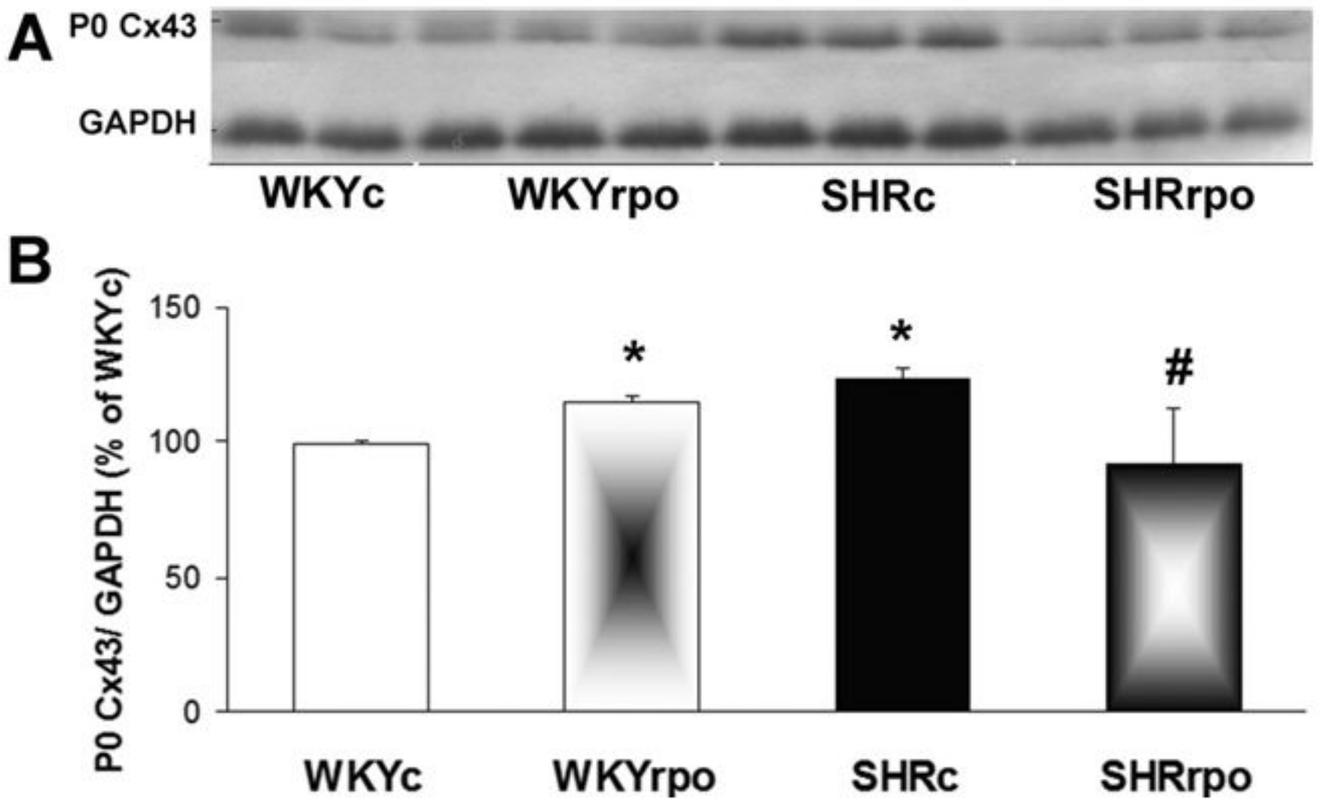


Fig. 4. Representative images of connexin-43 (Cx43) immunolabeling in the left ventricles of untreated and RPO-fed WKY and SHR rat hearts. Note the conventional distribution of Cx43-positive gap junctions predominantly at the intercalated discs (fine arrows) and sporadically on lateral surfaces (bold arrows) of the cardiomyocytes in both groups of WKY rats. The lateralization is enhanced in SHR hearts that additionally exhibit disordered expression of Cx43 at the intercalated disc (broken arrow). Supplementing the diet with RPO partially eliminated abnormal Cx43 distribution in SHR heart (SHRr). RPO, red palm oil; WKY, Wistar–Kyoto rats; SHR, spontaneously hypertensive rats. Magnification 40×.

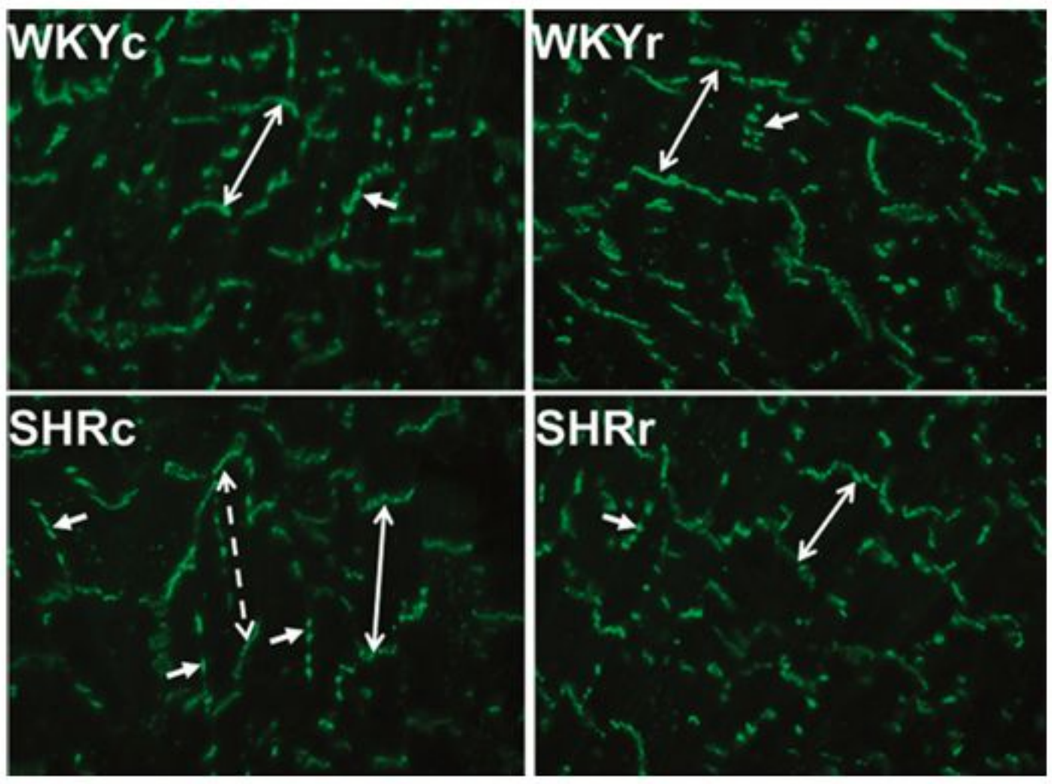
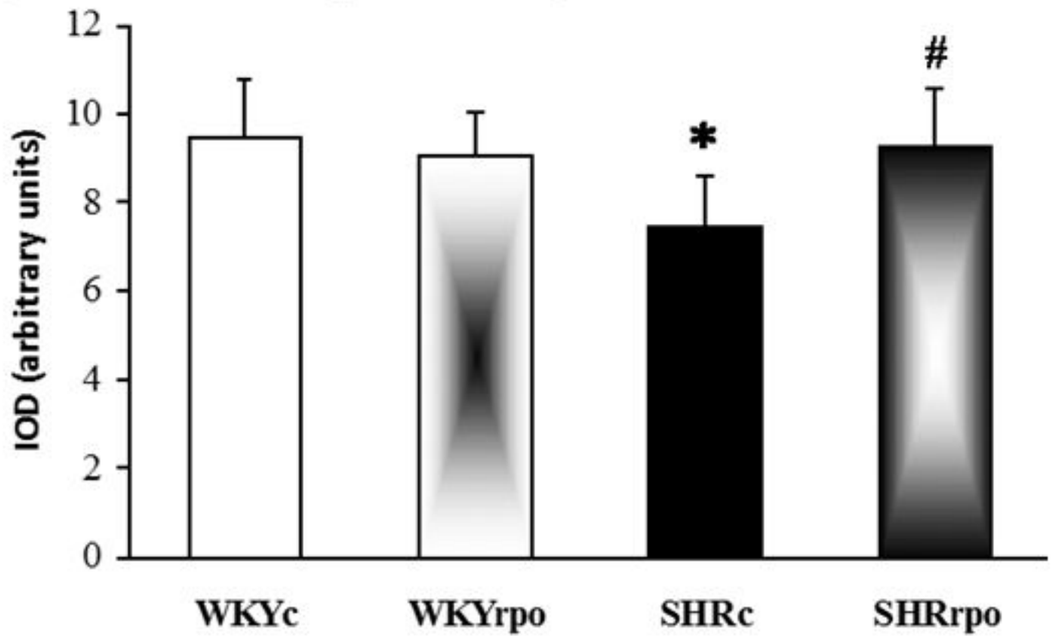


Fig. 5. Quntitative image analysis of connexin-43 (Cx43) immunoreaction of untreated and RPO-fed SHR and WKY rat heart ventricles. RPO, red palm oil; SHR, spontaneously hypertensive rats; WKY, Wistar–Kyoto rats; WKYc, control group (untreated) of WKY rats; WKYrpo, WKY rats fed RPO; SHRc, control group (untreated) of SHR; SHRrpo, SHR fed RPO. Results are the mean ± SD of 20 images per heart (8 hearts per group); *, $p < 0.05$ compared with WKYc; #, $p < 0.05$ compared with SHRc.



nate of SHR heart ventricle, while it did not reach a significant value in WKY rats (Figs. 6A and 6B). Notably, RPO intake did influence the translocation of PKCε and its distribution among the cellular fractions in SHR heart. There was an increase in PKCε expression in the cytosolic fraction and decrease in membrane-rich fraction. Data are plotted in Figs. 6C and 6D.

Incidence and duration of arrhythmias

Untreated SHR hearts exhibited a significantly shorter duration of early reperfusion-related bradycardia, compared with WKY rat hearts. However, the duration of bradycardia was markedly prolonged in SHR hearts after RPO supplementation, but not in WKY rat hearts (Fig. 7A). Furthermore, RPO intake almost eliminated early reperfusion-induced ventricular tachycardia (VT) in SHR (Figs. 7B and 7C), while RPO treatment had no effect on VT duration in WKY rat hearts, and even increased its incidence during 5 minutes of post-ischemic reperfusion (Figs. 7B and 7C). There was no difference in the incidence of electrically inducible sustained VF between WKY and SHR. However, more than 5 stimuli for the induction of sustained (2 min) VF were needed in WKY compared with 1–2 stimuli in SHR (not shown).

Fig. 6. Protein kinase Cε (PKCε) expression normalized to GAPDH and actin in the left ventricles of untreated and RPO-fed SHR and WKY rat hearts. (A) Immunoblot showing overall PKCε expression. (B) Densitometric quantification of PKCε expression. (C) Representative immunoblot of cytosolic (C) and particulate (P) fractions of PKCε as well as densitometric quantification of the expression. (D) C, open columns; P, filled columns. RPO, red palm oil; SHR, spontaneously hypertensive rats; WKY, Wistar–Kyoto rats; WKYc, control group (untreated) of WKY rats; WKYrpo, WKY rats fed RPO; SHRc, control group (untreated) of SHR; SHRrpo, SHR fed RPO. Results are the mean ± SD of 8 hearts per group; *, $p < 0.05$ compared with WKYc; #, $p < 0.05$ compared with SHRc.

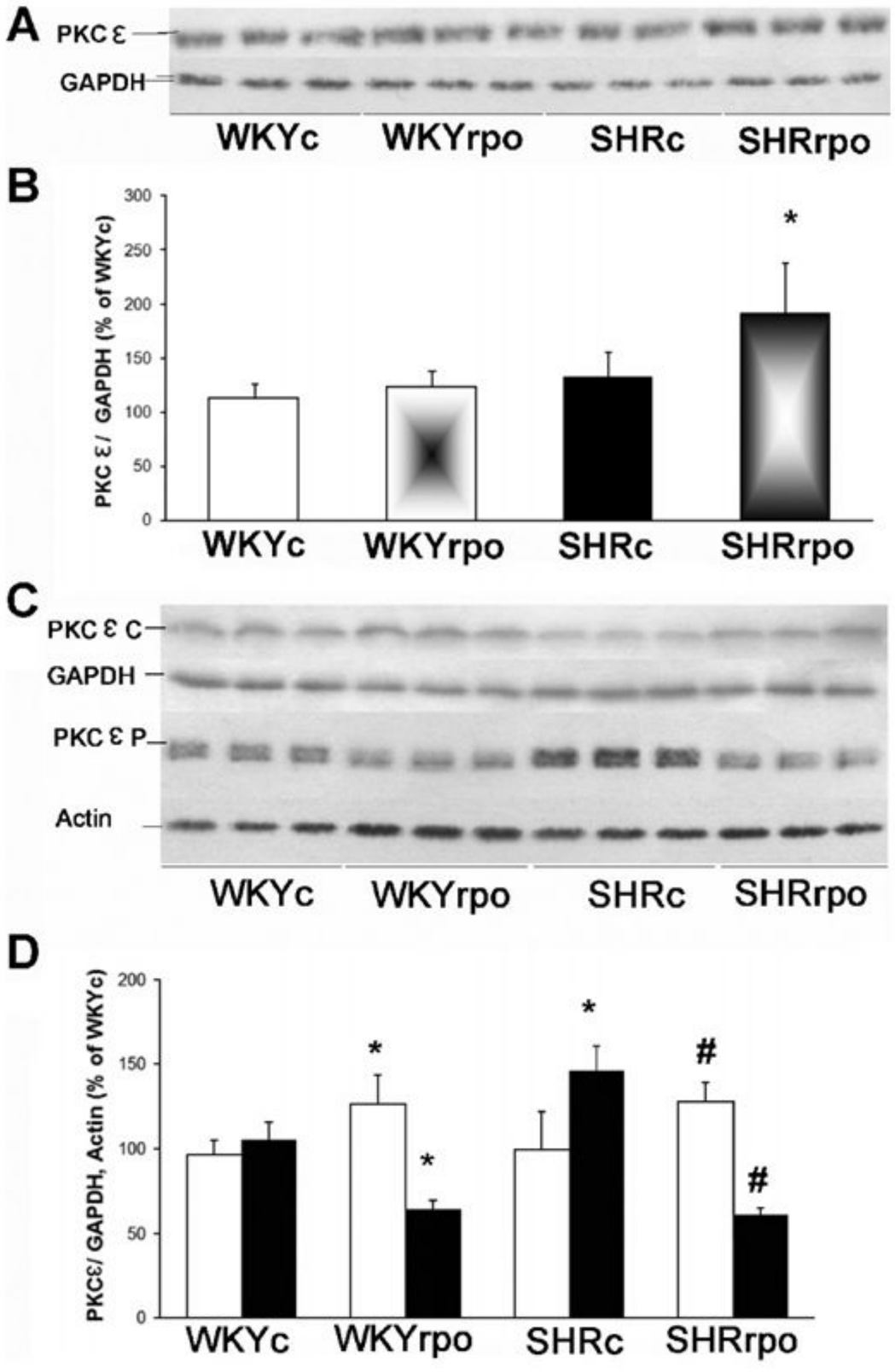
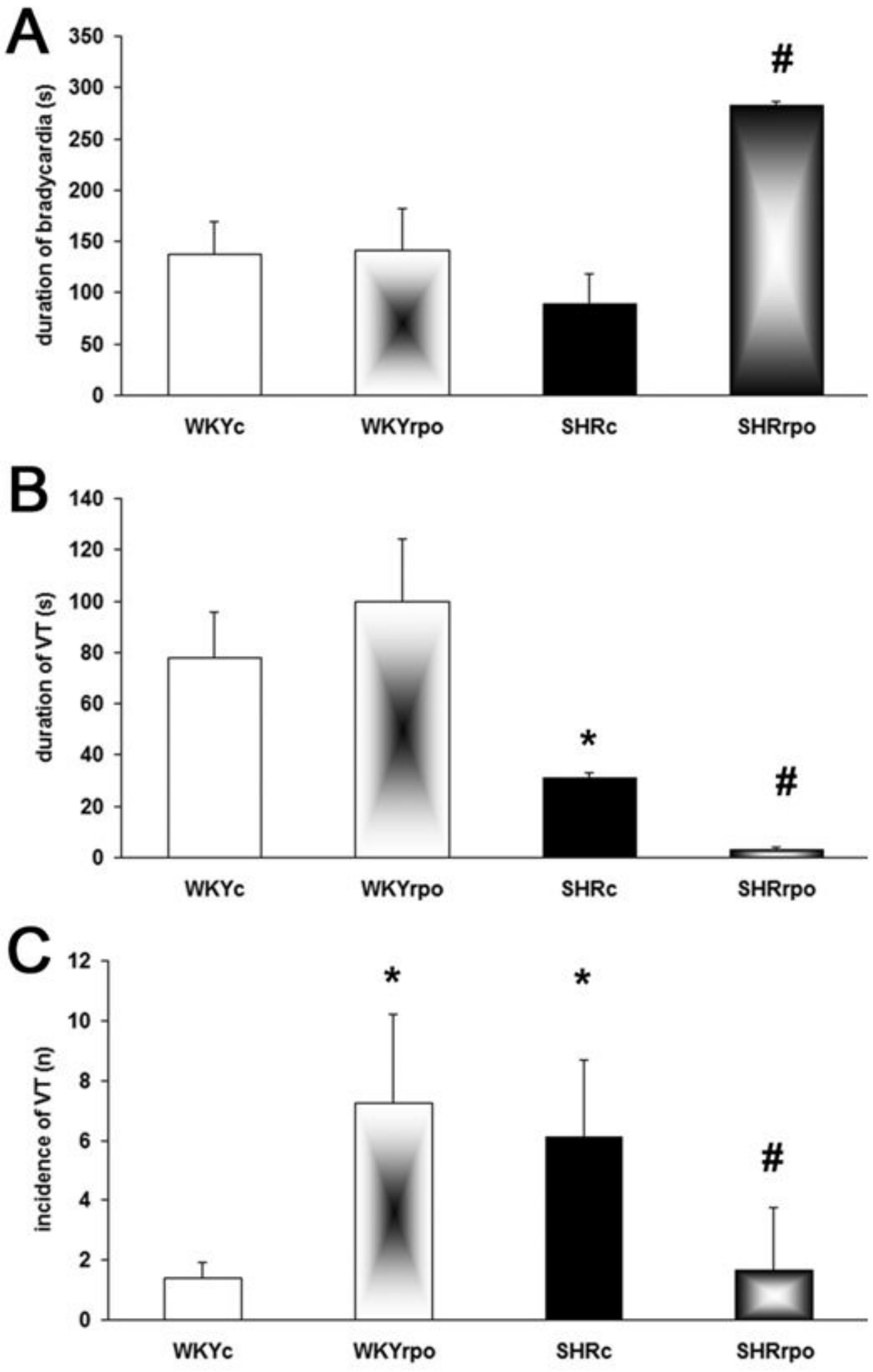


Fig. 7. (A) Duration of post-ischemic repefusion-induced bradycardia registered in untreated and RPO-fed SHR and WKY rat hearts. (B) Duration of post-ischemic repefusion-induced ventricular tachycardia (VT) registered in untreated and RPO-fed SHR and WKY rat hearts. (C) Incidence of post-ischemic repefusion-induced ventricular tachycardia (VT) registered in untreated and RPO-treated SHR and WKY rat hearts. RPO, red palm oil; SHR, spontaneously hypertensive rats; WKR, Wistar–Kyoto rats; WKYc, control group (untreated) WKY rats; WKYrpo, WKY rats fed RPO; SHRc, control group (untreated) SHR; SHRrpo, SHR fed RPO. Results are mean ± SD of 5 hearts per group; *, $p < 0.05$ compared with WKYc; #, $p < 0.05$ compared with SHRc.



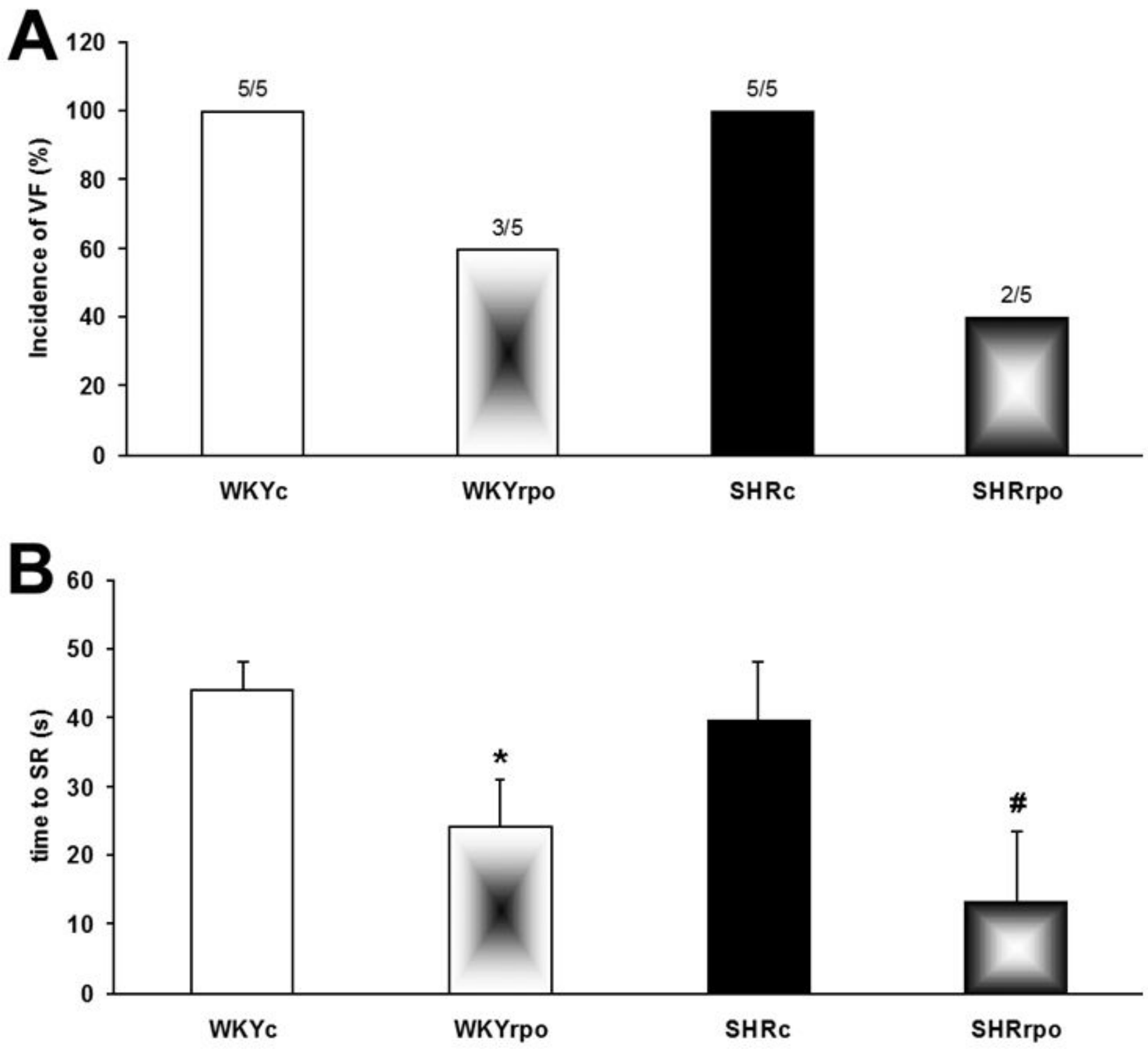
Supplementing RPO in the diet resulted in a clear reduction of inducible VF in SHR and, to lesser extent, WKY rats (Fig. 8A). Moreover, reversion of sustained VF into sinus rhythm was significantly faster in the hearts of RPO fed animals, namely in SHR and, to lesser extent, in WKY rats (Fig. 8B).

Discussion

We have demonstrated in this study, for the first time, that there is up-regulation of myocardial Cx43 and suppression of PKCε activation associated with ingestion of RPO by SHR. This was linked to the protection of the SHR heart from life-threatening arrhythmias. In addition, RPO supplementation reduced blood pressure in SHR, and blood glucose in both SHR and WKY rats. The cardioprotective effect of antioxidant-rich RPO in healthy WKY rat heart was, however, less pronounced, which may be partially attributed to the down-regulation of Cx43, although its cell membrane distribution was not altered.

Despite the progress in management of hypertension by the prevention or regression of myocardial remodeling (Paulis and Unger 2010; Simko and Pechanova 2010) there is still the need for novel therapeutic targets and approaches for cardiovascular risk reduction. Diet is very important in controlling blood lipids, whereby the amount and composition of fats present in the diet is one of the factors that plays a role

Fig. 8. (A) Incidence of electrically induced ventricular fibrillation (VF) registered in untreated and RPO-fed SHR and WKY rat hearts. Numbers of fibrillating hearts to total number of hearts per group are demonstrated. (B) Time to reversion of VF into sinus rhythm registered in untreated and RPO-fed SHR and WKY rats. RPO, red palm oil; SHR, spontaneously hypertensive rats; WKY, Wistar–Kyoto rats; WKYc, control group (untreated) WKY rats; WKYrpo, WKY rats fed RPO; SHRc, control group (untreated) SHR; SHRrpo, SHR fed RPO. Results are the mean ± SD of 5 hearts per group; *, $p < 0.05$ compared with WKYc; #, $p < 0.05$ compared with SHRc.



in the regulation of BP and incidence of hypertension. Accordingly, saturated fats and oxidized oils cause an increase in BP, while ingestion of unsaturated fats has the opposite effect. It has been shown that diets containing 15% (*w*/*w*) RPO for 18 weeks did not raise BP (Osim et al. 1996). We have demonstrated both antihypertensive and hypoglycemic effects resulting from RPO supplementation. It appears that RPO, in addition, may affect insulin resistance and (or) glucose metabolism. This needs to be explored more in detail.

SHR were used in this study to mimic human essential hypertension that is known to be associated with increased risk for lethal arrhythmias, particularly due to structural remodeling of the heart. Left ventricular hypertrophy (LVH) can be considered as an adaptive process serving to compensate for haemodynamic load. However, LVH is accompanied by abnormal distribution (lateralization) of Cx43, which can contribute to the development of arrhythmogenic substrate (Dupont et a. 2001; Tribulová et al. 2002; Kostin et al. 2003, 2004; Mitasíková et al. 2009; Bacova et al. 2010; Radošinská et al. 2011). Indeed, as we have shown in this and previous studies (Tribulová et al. 2002*a*, 2003), abnormal topology (remodeling) and down-regulation of gap junction Cx43 are linked with increased susceptibility of the SHR heart to malignant arrhythmias. Accordingly, SHR exhibited either increased incidence of VT and VF, or an increased number of stimuli was needed to induce VF. Although it is difficult to prove that altered Cx43 expression and (or) distribution causes arrhythmias and heart failure, the substantial evidence, including Cx43 knockout models in mice, indicates that Cx43 alterations play an important pathogenic role (Peters and Wit 1998; Saffitz 2006; Severs et al. 2008, Tribulová et al. 2008; Mitasíková et al. 2009). Results of this study support this view.

On the other hand, our findings demonstrated that feeding of SHR with RPO for 5 weeks results in a significant myocardial increase of both Cx43-mRNA and Cx43 protein levels as well as its functional phosphorylated forms. Interestingly, we have recently found similar up-regulation of Cx43 in the heart of spontaneously diabetic rats fed omega-3 FA (Radošinská et al. 2010). Whether the unsaturated FAs or the other FAs present in RPO are involved in modulation of Cx43 expression is not clear yet. However, it has been reported that various cardioprotective drugs and FAs, such as statins, sartans, antidiabetics, and omega-3 FA, are ligands for peroxisome proliferator-activated receptor (PPAR), and consequently activate specific gene expression (Marx, et al. 2003; Shen et al. 2010; Maejima et al. 2011). Accordingly, we hypothesize that some compound present in RPO may, via PPAR, affect *Cx43* gene transcription. Indeed, direct transcriptional activation of Cx43 mRNA by carotenoids (component of RPO) has been reported in an experimental model of cancer (Bertram and Vine 2005). Further studies should

explore the impact of individual anitioxidants, e.g., tocotrienols (Das et al. 2008) and FAs present in RPO on *Cx43* gene and protein expression. Perhaps it might also elucidate why Cx43 mRNA was suppressed in healthy RPO-treated WKY rat heart. We have demonstrated that not only the expression, but also phosphorylated (functional) forms of Cx43 are enhanced owing to RPO supplementation in SHR heart ventricle. In contrast, this was suppressed in RPO-treated WKY rat heart, which in addition exhibited an increase of unphosphorylated (nonfunctional) form of Cx43. It is, however, expected that the "treatment" dose of RPO may result in differential response of healthy WKY rats with normal cardiac intracellular and intercellular signaling compared with diseased SHR, which suffer from numerous cardiac abnormalities including a deficiency of membrane α-tocoferol (Janero and Burghardt 1989). This is consistent with recent findings showing differential dose-related effects of tocotrienols (Das et al. 2008). Thus, it may be argued that an "overdose" of antioxidants may have undesirable effects in healthy individuals. Consequently, this suggests that there would be differences between the effective dose of RPO to prevent CVD, and the dose to treat individuals who suffer from disease-related oxidative stress and abnormal lipid metabolism. Moreover, RPO, owing to its antioxidative properties, may reduce Cx43 degradation, which is normally enhanced by free radicals (Smyth et al. 2010).

In parallel, the expression of PKCε, which is known to phosphorylate Cx43, was significantly enhanced in RPO-treated SHR, but not WKY rat heart. However, an increase in PKCε expression was related to its cytosolic fraction (due to its internalization) and not its membrane fraction. This indicates that RPO intake results in an increase of PKCε pool, but not activity, which could be responsible for enhanced Cx43 phosphorylation. Enhanced cytosolic pool can be activated (by translocation into cell membrane) owing to acute pathological conditions such as ischemia or reperfusion. Several other kinases such as extracellular signal-regulated kinase (ERK), tyrosine kinase src, casein kinase 1, protein kinase A (PKA), protein kinase B (Akt), and protein kinase G (PKG) have been implicated in Cx43 phosphorylation (Jeyaraman et al. 2012). Activation of the JNK pathway down-regulates Cx43 (Petrich et al. 2002), while suppression of this pathway by RPO (Engelbrecht et al. 2006) may protect Cx43. Previous studies have also suggested that RPO supplementation most likely up-regulates NO–cGMP–PKG signaling (Esterhuyse et al. 2006), which is known to affect myocardial Cx43 channel function (Salameh and Dhein 2005). The role of eNOS/iNOS in the modulation of Cx43-mediated gap junctional communication in SHR hearts has been suggested recently (Radošinská et al. 2011). In the normal heart, Cx43 is phosphorylated at multiple sites resulting in electrophoretic migration at 45 kDa, with fully unphosphorylated Cx43 at 41 kDa. Of note, the factors and conditions altering the phosphorylation pattern of Cx43 can also alter its properties (electrical and metabolic coupling), and consequently affect both heart function and its susceptibility to arrhythmias (Salameh and Dhein 2005; Jeyaraman et al. 2012). Indeed, our results show that enhanced myocardial expression and phosphorylation of the Cx43 in RPO-treated SHR is linked with protection from post-ischemic-reperfusion-induced VT and electrically inducible VF. However, there was no VT prevention during reperfusion in RPO-treated WKY rats that exhibited decrease of both expression and phosphorylation of Cx43. Interestingly, there was some protection from electrically induced VF, likely because cell membrane distribution of Cx43 was not altered in the RPO-treated WKY rat hearts.

In the context of possible modulation of Cx43-mediated cell-to-cell communication by RPO, there are additional factors that may play a fundamental role. As with other ion channels, Cx43 channel function and assembly of connexin into gap junctions can be modulated by the composition of the lipid bilayer (Cascio 2005). It is consistent with our findings that not only down-regulation of Cx43 but also its disordered myocardial distribution was attenuated in SHR fed with RPO, as revealed in in-situ immunolabeling. Omega-3 FA and the antihypertesive drug aliskiren, have also been reported to restore normal localization of Cx43, enhance Cx43 expression, and attenuate electrical remodeling and arrhythmia induction in a transgenic rat model of high renin hypertension (Fischer et al. 2008). It is very likely that attenuation of abnormal Cx43 expression and distribution by RPO can be beneficial for myocardial cell-to-cell communication in SHR heart to improve its synchronization. This may explain, at least partially, the reduction of electrically induced sustained VF and the incidence of post-ischemic reperfusion-induced VT. In addition, high levels of Cx43 in RPO-treated SHR most likely contributed to the enhanced ability of the heart to terminate VF and to restore sinus rhythm. The link between up-regulation of Cx43 and decreased susceptibility of the heart to malignant arrhythmias is supported by the fact that down-regulation of Cx43 in WKY was associated with much reduced arrhythmia protection.

Taken together, despite some limitations of the study dealing with number of rats for examining arrhythmias, our findings indicate that SHR benefit from RPO intake, particularly owing to up-regulation of myocardial connexin-43 associated with apparent anti-arrhythmic effects. Moreover, it appears that the RPO dose should be adjusted according to whether it is being used for the treatment or the prevention of heart disease. It is promising that RPO supplementation, likewise to omega-3 FA intake, in combination with antihypertensive drugs may reduce the risk from malignant arrhythmias and heart failure in patients suffering from hypertension via up-regulation of Cx43. To support this idea, further studies are needed to elucidate which component of RPO is involved, and how it is involved in the regulation of Cx43 expression and modulation of cell-to-cell coupling to protect myocardial synchronization.

Conflict of interest

The authors delare that there are no conflicts of interest or disclosures associated with this study.

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