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RESEARCH ARTICLES

Alcohol-free red wine inhibits isoproterenol-induced cardiac remodeling in rats by the regulation of Akt1 and protein kinase C α/β II☆

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Abstract

There is increasing evidence that moderate consumption of red wine containing high amount of polyphenols and anthocyanins is associated with decreased incidence of cardiovascular morbidity and mortality. Therefore, we hypothesized that cardiac hypertrophy and fibrosis as well as Akt (protein kinase B, PKB) and protein kinase C (PKC) cascades can be beneficially influenced by an alcohol-free red wine (AFRW) extract rich in 14 types of polyphenols and 4 types of anthocyanins during cardiac remodeling. To test this assumption, rats were treated with isoproterenol (ISO) to induce postinfarction remodeling and were given tap water or AFRW *ad libitum* for 8 weeks. Control rats received vehicle instead of ISO. Heart mass/body mass and ventricle mass/body mass ratios, diameter of cardiomyocytes, phosphorylation of PKC α/β II and protein kinase B/Akt, and deposition of collagen type III were determined from the hearts of all four groups of rats. All measured gravimetric parameters, myocyte diameters and the amount of collagen type III decreased, and the phosphorylation of PKC α/β II was reduced in the ISO+AFRW group compared to the ISO group. AFRW induced activation of Akt, one of the best characterized cytoprotective pathways even without ISO treatment, and this activation was further increased in the ISO+AFRW group. These data suggest that AFRW treatment has a protective effect on hearts undergoing postinfarction remodeling by repressing hypertrophy-associated increased phosphorylation of PKC alpha/beta II and by activating Akt, providing a molecular mechanism for the cardioprotective effect of red wine polyphenols.

Keywords: Alcohol-free red wine; Cardiac remodeling; Intracellular signaling pathways; Cardiac fibrosis

1. Introduction

Epidemiological data suggest that moderate alcohol consumption is associated with decreased incidence of cardiovascular morbidity and mortality. Consumption of red wine rich in different types of polyphenols and anthocyanins has been reported to provide greater benefit in the prevention of cardiovascular diseases than the consumption of other alcoholic beverages. Mortality levels provoked by cardiovascular diseases are much lower in France than in other industrialized countries, even though the consumption of saturated fats is as high and blood cholesterol level is generally higher in France than in the other developed countries ("French Paradox," World Health Organization, 1989). Furthermore, other factors associated with risks of chronic heart failure (CHF), such as arterial blood pressure and smoking, are also as high in France as in the other countries [1]. Recently, understanding of the French paradox has encouraged new research to reveal whether polyphenol antioxidants may have protective effects on the cardiovascular system [2].

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Experimental models of CHF can be induced by several methods. Subcutaneous administration of beta-adrenoceptor agonist isoproterenol (ISO) produces patchy myocardial necrosis in a dose-related way with intact coronary vasculature.

Acute myocardial infarction triggers left ventricular (LV) remodeling characterized by necrosis and thinning of the infarcted myocardium, LV chamber dilatation, fibrosis both at the site of infarct and in the noninfarcted myocardium, and hypertrophy of viable myocytes. Pathologic hypertrophy following myocardial infarction often progresses into heart failure, which is one of the leading causes of morbidity and mortality worldwide and characterized by a progressive deterioration in heart function [3]. Although early remodeling may be adaptive and sustain LV function in the short-term, persistent remodeling may contribute to functional decompensation [3,4].

Cardiac remodeling is characterized by inadequate myocyte hypertrophy and accumulation of extracellular matrix structural proteins (collagen type I and III), which may contribute to myocardial stiffness by limiting the motion of myocytes and may promote arrhythmias by electrical isolation of adjacent myocytes [5]. Additionally, tissue stiffness ultimately leads to diastolic and systolic dysfunction, while perivascular fibrosis and disorganized hypertrophy can exhaust the coronary blood flow reserve and consequent hypoperfusion may provoke myocardial ischemia [5].

The polyphenol fraction of wines includes phenolic acids, trihydroxy stilbenes, oligomer proanthocyanidines and flavonoids, all of them functioning as antioxidants and have been reported to be cardioprotective in various ischemic heart diseases [1,6].

trans-Resveratrol (3,4′,5-*trans*-trihydroxystilbene) is a natural phytoalexin produced in a variety of plant species including grapes and grape products such as wine [7–9]. There are increasing evidences that *trans*-resveratrol has positive effects on both human and animal health. It increases life span and improves mitochondrial function and metabolic homeostasis in lower organisms and in mice [10,11]. It can decrease lipid peroxidation, cholesterol and triglyceride deposition, and inhibit vascular smooth muscle cell proliferation and migration, tumor initiation and progression [12]. It up-regulates NO production, prevents low-density lipoprotein oxidation [12–14] and increases the expression of LDL receptors. Additionally, it has been reported that the ischemic damage to the myocardium is significantly lower in chronic pretreated rats than in the nontreated group [7,15].

Other polyphenols, which occur also in red wines, were shown to have beneficial effects, too. Quercetin has been inversely linked to mortality from coronary heart disease (CHD), because of its antioxidant capacity *in vitro* and *in vivo*. It was also proven that quercetin can modulate gene expression through inhibition of signal transduction pathways including protein kinase C (PKC) and may lead to stabilization of atherosclerotic plaques [16].

Catechins and procyanidins reduce the formation of reactive oxygen species in the ischemic-reperfused myocardium. Procyanidins provide cardioprotection by antagonizing the ischemia-reperfusion induced activation of c-Jun N-terminal kinase (JNK) and c-Jun [17], while catechins inhibit platelet aggregation and may prevent chronic inflammatory diseases [18].

The exact role of the ethanol component is still unclear. Epidemiological studies revealed that besides moderate alcohol consumption (two glasses of wine or two glasses of beer per day), incidence of CHD is lower than in the group of abstinents [19,20], while incidence of CHD in alcohol abusers is significantly higher [21]. Ethanol may facilitate cellular uptake of polyphenols [22]. In the case of ischemia-reperfusion injury in rats, the ethanol-treated group displayed slightly better functional recovery, which deteriorated sharply toward the end of the reperfusion period and the extent of infarction was decreased.

Several intracellular signaling pathways have been proven to play important roles in the development of cardiac hypertrophy during postinfarction cardiac remodeling. Cells of the cardiovascular system respond to a variety of physiologically important stimuli, cytokines and stresses. Protein kinase C, a member of the serine–threonin family, is a key enzyme for various cellular functions including cell growth, apoptosis and differentiation. In cardiomyocytes, numerous hypertrophic stimuli such as angiotensin II, endothelin-1, β-adrenergic agonists and mechanical stress have been shown to activate various PKC isoforms [23].

Akt (protein kinase B, PKB) may promote myocyte survival and can be activated by the Ras subfamily and MAPKs [24]. MAPK subfamilies have been implicated in the response to stresses which includes hyper- and hypo-osmotic shock, physical stretch or deformation, increased rates of contraction (pacing), exposure to reactive oxygen species (oxidative stress), chemical stresses and shear stress. A wide variety of growth-promoting/hypertrophic agents activates these kinases in cardiac myocytes, fibroblasts, smooth muscle cells or endothelial cells [25].

Therefore, the aims of our investigation were to reveal the effects of an alcohol-free red wine (AFRW) on Akt/PKB and PKC α/β II pathways and to study whether it can attenuate cardiac hypertrophy and the deposition of collagen type III in rats in the case of intact heart and postinfarction remodeling.

2. Materials and methods

2.1. Materials

Red wine was vacuum distilled at 36°C until the alcohol component disappeared, acid content was absorbed by 1 g/L calcium carbonate and volume was increased five times with tap water to obtain AFRW.

trans-Resveratrol standard (99%) was purchased from Sigma-Aldrich Co. (Budapest, Hungary), *trans*-piceid (β-glycosid isoform of *trans*-resveratrol) standard from

Herbstandard, Inc. (Chesterfield, MO, USA), acetic acid (96%) from Riedel-de Haën GmbH and Co. (Seelze, Germany) and methanol [high-performance liquid chromatography (HPLC) grade] from Scharlau Chemie SA (Barcelona, Spain). All other chemicals were of analytical grade. Freshly bidistilled water was used for the preparation of the aqueous solutions. Wine samples were a kind gift of Polgar Winery (Villany, Hungary).

Standards of polyphenols were dissolved in 1 cm^3 of ethanol and filled up with the eluent. Wine samples were directly injected, without pretreatment. All standard solutions and wine samples were stored in the dark at 5°C to avoid oxidative degradation and isomerization of the *trans*-resveratrol and *trans*-piceid to *cis*-forms.

2.2. Animals

Male CFY-strain Sprague-Dawley rats (300–350 g) were used for the experiments. Animals received human care according to the Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health (NIH Publication No. 85-23, revised 1996), and the experiment was approved by the Animal Research Review Committee of the University of Pecs, Medical School. The animals were housed under standardized conditions, 12-h dark–light cycle in solid bottomed polypropylene cages, and received commercial rat chew *ad libitum*. AFRW or tap water was administered as drink *ad libitum* for 8 weeks.

We set the dosage of AFRW and resveratrol based on average moderate daily consumption of wine for human, namely, about 250 ml of wine corresponding to 0.018 mg/kg resveratrol. Unexpectedly, the rats consumed more AFRW than tap water, so the actual dosage was 0.069 mg/kg resveratrol for our animals.

2.3. Cardiac remodeling

To induce postinfarction remodeling, the rats were treated two times on two consecutive days with 80 mg/kg ISO (Sigma-Aldrich) or vehicle subcutaneously as previously described [3]. The animals were divided into four groups: control group (n=10), watered with tap water without ISO treatment; ISO group (n=10), watered with tap water with ISO treatment; ISO+AFRW group (n=10), watered with AFRW with ISO treatment; AFRW group (n=10), watered with AFRW without ISO treatment.

Eight weeks after ISO challenge, body weight was measured, animals were sacrificed and the hearts were removed. Atria and great vessels were trimmed from ventricles; weight of ventricles was measured and normalized to body mass. Afterwards, ventricles were fixed in 10% formalin for histology or freeze clamped for Western blot analysis.

2.4. Analytical studies

The polyphenol and anthocyanin contents of AFRW samples were determined by HPLC consisting of a Gynkotek

Table 1
Types and exact concentrations of polyphenols and anthocyanins of AFRW

A. Polyphenol contents of AFRW													
Gallic acid	Tyrosol	Caftaric acid	Catechin	GRP	Procyanidin B2	Caffeic acid	Epicatechin	*p*-Coumaric acid	Fretaric acid	Rutin	Ferulic acid	*trans*-Resveratrol	*trans*-Piceid	Quercetin
65.9±3.7	81.2±1.9	51.6±9.8	89.1±3.0	2.5±0.2	47.5±3.6	18.5±1.1	126±9.1	10.2±1.3	3.0±0.5	16.9±1.2	2.6±0.4	3.9±1.0	6.01±1.6	11.2±1.9

B. Anthocyanin contents of AFRW				
Delphidin-3-glucoside		Petunidin-3-glucosie	Peonidin-3-glucoside	Malvidin-3-glucoside
53.7±5.3		49.6±6.9	38.2±7.7	276±26

Quantitative and qualitative analysis of AFRW samples was performed by HPLC-coupled mass spectrometer (see details in Materials and Methods). Results are expressed as mean±S.E.M. (mg/L) (n =3).

M 580 GT pump, a Rheodyne 8125 (20-µl loop) injector and a Gynkotek M 340S UV diode array detector (Gynkotek GmbH, Germering, Germany). A 250×4.6-mm column, packed with 5-µm particle size Kovasil-based C_{30} material, has been used for the separations. The Chromeleon data management software (Dionex Corp., Sunnyvale, CA, USA) was used for the control of the equipment and for data evaluation. Quantification was carried out using the peak area method. A multistep gradient method was applied using methanol–water–acetic acid (10:90:1 V/V) mixture as solvent A and methanol–water–acetic acid (90:10:1 V/V) mixture as solvent B at a flow rate of 1.5 cm^3/min. The gradient profile was 0.0–18.0 min from 0% to 40% B; 18.0–25.0 min from 40% to 100% B; 25.0–27.0 min 100% B. Chromatographic separations were monitored at 306 nm. Chromatographic peaks were identified by comparing retentions and UV spectra and MS spectra of the samples with those of standard compounds. Quantification was carried out by external standardization.

Mass spectrometry (MS) analysis was performed using a Finnigan AQA (Thermoquest, San José, CA, USA) mass spectrometer equipped with both atmospheric pressure chemical ionization (APCI) and electrospray ionization (ESI) interfaces. Both the auxiliary and curtain gas were nitrogen at a flow rate of 600 L/h. For LC-MS analysis, we used APCI and ESI ionization source, probe temperature was 250°C, corona voltage or probe voltage was 3.5 kV. Spectra were recorded at a rate of 1.2 scans/s in the negative ion mode between *m/z* 10 and 700. Scan filter on the quadrupole analyzer was 10 and 20 V. Finnigan Xcalibur (version XCALI-97006) was used to acquire mass spectra of the compounds.

For the types and exact concentrations of polyphenols and anthocyanins in AFRW see Table 1.

2.5. Histology

Histological analysis was performed as described previously [3]. Briefly, mean myocyte diameters on picrosirius red-stained sections were calculated by measuring 100 cells per specimen using the two-point distance function of the TelPath analyzer system (www.bollmann.com, 2000). Collagen type III was stained as a marker of interstitial fibrosis on frozen sections, 5 µm thick, by the Vector M.O.M. Kit (Vector Laboratories, Inc., Burlingame, CA, USA) staining procedure.

Primary mouse antisera against collagen type III (1:1000, Monoclonal Anti-collagen, Type III, Sigma-Aldrich) diluted in M.O.M. diluent was reacted at room temperature for 30 min, followed by two 2-min rinses in TBS. Biotinylated anti-mouse IgG reagent was then applied for 10 min and sections were washed twice for 2 min in TBS. VECTASTATIN ABC Reagent was applied for 5 min followed by two 2-min rinses in TBS. Sections were then stained with Vector NovaRED Substrate (Vector Laboratories) for 5 min, washed in distilled water, dehydrated and mounted on slides. Sections were quantified with the NIH ImageJ analyzer system.

2.6. Western blot analysis

Western blotting was performed as previously described [3]. Membranes were probed overnight at 4°C with antibodies recognizing the following antigens: phospho-specific Akt-1/PKB-$\alpha^{(Ser\ 473)}$ (1:1000) (Sigma-Aldrich) and phospho-specific PKC α/β $II^{(Thr\ 638/641)}$ (1:1000) (Cell Signaling Technology, Beverly, MA, USA). Goat anti-rabbit horseradish peroxidase-conjugated secondary antibody was used (1:3000 dilution; Bio-Rad, Budapest, Hungary) and antibody–antigen complexes were visualized by enhanced chemiluminescence. Results of Western blots were quantified by the NIH ImageJ program.

2.7. Statistical analysis

Data are expressed as means±S.D. Statistical analysis was performed by two-tailed and two-sample Student's *t*-test; *P* values below .05 were considered significantly different.

3. Results

3.1. AFRW Treatment improves gravimetric parameters in ISO-induced remodeling

In our investigation, gravimetry performed 8 weeks after ISO-induced myocardial infarction showed significantly elevated heart mass normalized to body mass (control: 2.36±0.05

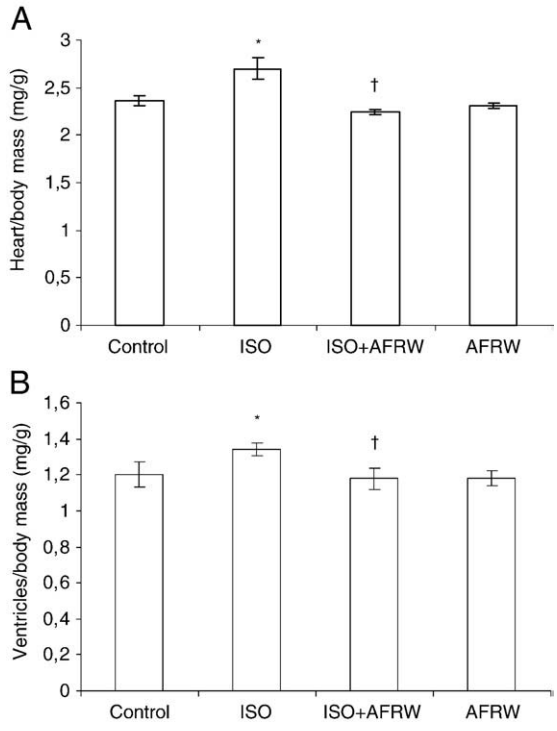

Fig. 1. Effects of AFRW and ISO on the gravimetric parameters. Eight weeks after ISO-induced CHF, mass of hearts and ventricles were measured, and the results were expressed as mean±S.E.M. (mg/g) (n=10) for heart/body mass ratio (A) and ventricle/body mass ratio (B). *Significantly different from control, $P<.05$. †Significantly different from ISO, $P<.05$.

vs. ISO: 2.7±0.11 and ISO+AFRW: 2.47±0.025 vs. ISO: 2.7±0.11, $P<.05$). AFRW treatment significantly prevented these unfavorable changes. Furthermore, AFRW treatment reduced ISO-induced ventricular mass gain (ISO+AFRW: 1.15±0.06 vs. ISO: 1.34±0.035, $P<.05$). In the AFRW group, heart mass and ventricle/body mass ratio were not influenced by AFRW compared to the control group (control ventricle/body mass: 1.2±0.07 vs. AFRW ventricle/body mass: 1.18±0.05, control heart/body mass ratio: 2.36±0.05 vs. AFRW heart/body mass ratio: 2.38±0.06, $P>.05$; Fig. 1).

3.2. AFRW Moderates myocardial hypertrophy and interstitial collagen type III deposition

Myocardial hypertrophy was characterized by increases in both cell length and diameter. Histological analysis revealed that AFRW treatment significantly attenuated ISO-induced mean myocyte diameter increase ($P<.05$; Fig. 2). The diameter of the myocytes in the AFRW-treated group remained unaltered compared to the control group (picture not shown).

ISO-induced interstitial collagen type III deposition was significantly decreased in the ISO+AFRW-treated group ($P<.05$; Fig. 3), while it was not attenuated in the AFRW-treated group compared to the control group ($P>.05$; picture not shown).

3.3. AFRW Reduces PKC α/β phosphorylation in failing myocardium

The moderate PKC α/β II phosphorylation present in control samples increased in the ISO-treated group. Enhanced phosphorylation of PKC α/β II seen in post-infarcted samples was significantly attenuated by AFRW treatment (control vs. ISO, $P<.01$; ISO+AFRW vs. ISO, $P<.01$; Fig. 4A,C).

3.4. AFRW Influences the phosphorylation of Akt

The moderate Akt-1 phosphorylation in the control group was increased in the ISO-treated animals (control vs. ISO, $P<.01$) and became strongly phosphorylated in the ISO+AFRW-treated group (ISO vs. ISO+AFRW, $P<.01$). The activity of Akt-1 was also enhanced in the AFRW group compared to the control group (control vs. AFRW, $P<.01$; Fig. 4B,C).

4. Discussion

In our study, we investigated the effect of AFRW on ISO-induced postinfarction cardiac remodeling as well as on healthy hearts in rats.

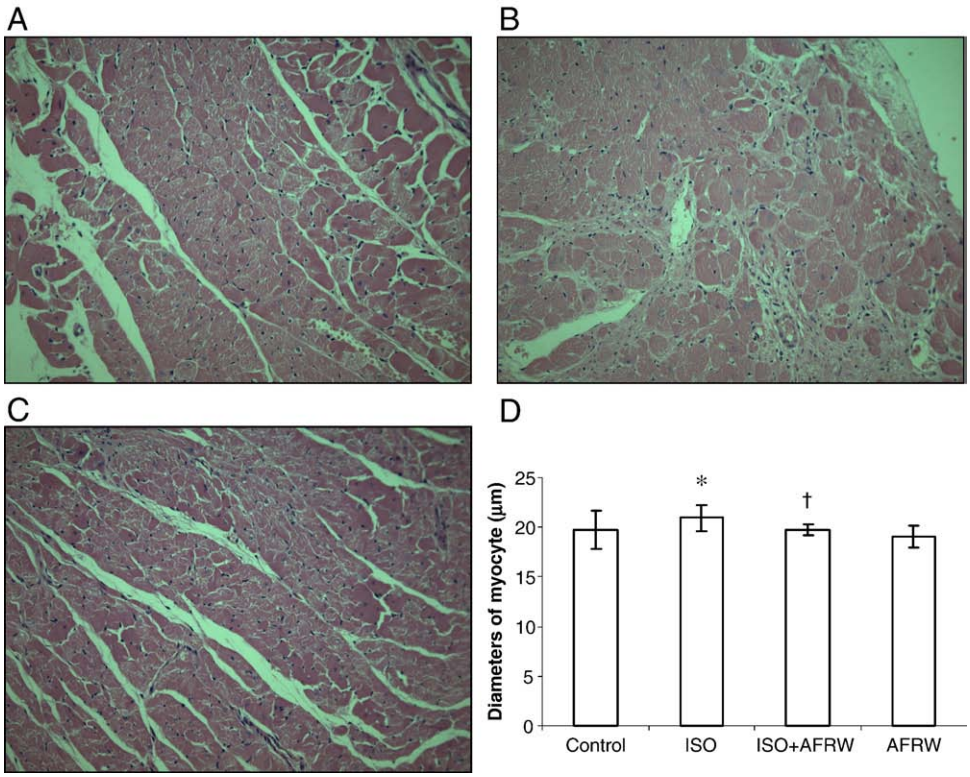

Fig. 2. Effects of AFRW and ISO on myocardial hypertrophy. Eight weeks after ISO-induced CHF, heart sections were stained with hematoxylin and eosin, and mean myocyte diameters were measured for 100 cells per section and expressed as mean±S.E.M. (μm) (n=10) (D). Representative photos for 10 animals per group are presented for control (A), ISO- (B) and ISO+AFRW (C)-treated groups. Hearts from the AFRW-treated group were identical to control (data not shown). *Significantly different from control, $P<.05$. †Significantly different from ISO, $P<.05$.

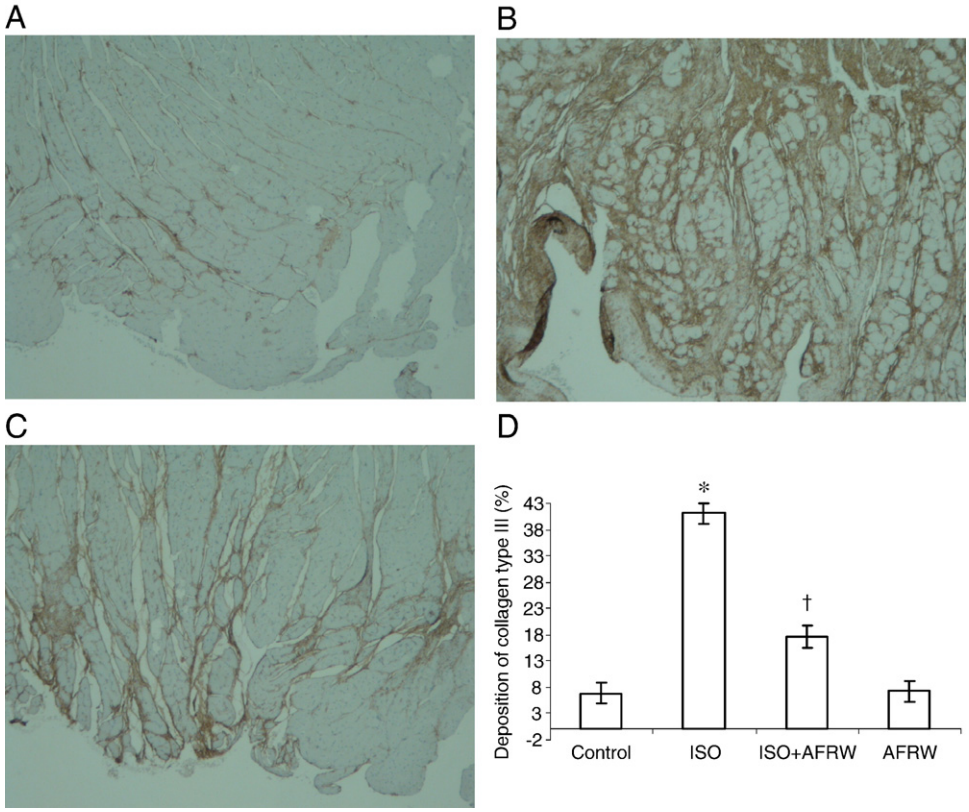

Fig. 3. Effects of AFRW and ISO on interstitial collagen type III deposition. Eight weeks after ISO-induced CHF, heart sections were immunostained with an antibody against type III collagen, immunopositive areas/section were densitometrically evaluated and expressed as % mean±S.E.M. (n=10) (D). Representative photos for 10 animals per group are presented for control (A), ISO- (B) and ISO+AFRW (C)-treated groups. Hearts from the AFRW-treated group were identical to control (data not shown). *Significantly different from control, $P<.05$. †Significantly different from ISO, $P<.05$.

Isoproterenol produces patchy myocardial necrosis in a dose-related way with an intact coronary vasculature. Administration of the drug produces graded myocardial cell death and rapidly impairs left ventricular function, at least partially, through free-radical generation [26]. Pathologic hypertrophy following myocardial infarction often progresses into heart failure, which is one of the leading causes of morbidity and mortality worldwide and characterized by a progressive deterioration in heart function.

After myocardial infarction, initial loss of myocardium can induce progressive ventricular enlargement and deposition of interstitial deposition of collagen type III, both changes are intended to adapt to the altered situation. However, increasing muscle mass is achieved by enlarging cardiomyocytes rather than increasing their number, resulting in reduced heart performance and, together with the increased collagen deposits, decreased elasticity. In this experiment, heart mass/body mass and ventricle/body mass ratios were significantly attenuated by AFRW; moreover, AFRW treatment significantly reduced myocardial hypertrophy and interstitial deposition of collagen type III in remodeling hearts, and these parameters were not influenced in the group treated with AFRW alone. All these effects of AFRW lead to increased performance of the postinfarct heart. Furthermore, these effects were necessarily due to one or more of the polyphenol and/or anthocyanin components of AFRW, since AFRW did not contain any other substances of possible biological effect.

A number of studies have proven that alterations of intracellular signaling pathways play a crucial role in the development of cardiac hypertrophy, which is induced and maintained by vasoactive peptides, peptide growth factors, hormones and neurotransmitters, which all act upon PKC and MAPK pathways [25]. PKC has long been implicated in cell proliferation, survival and programmed cell death [12]. Studies of myocardial hypertrophy and heart failure report similar findings: PKCα and PKCβ are up-regulated via growth factor receptor phosphorylation or lipid secondary messengers (derived from the reactions of other receptor-regulated enzymes such as phospholipases A2, D and C) [26]. Therefore inhibition of PKC may be a therapeutic target in cardiac remodeling. We found that 8 weeks after myocardial infarction, AFRW attenuated ISO-induced PKC α/β II phosphorylation, although the mechanism by which AFRW diminished the activity of PKC α/β II still needs to be elucidated.

It was shown that *trans*-resveratrol can influence the activation of MAPK cascades [21,27,28] and the survival of kinase Akt in intact and isolated hearts [29,30]. Since Akt activation was shown to antagonize pathologic cardiac

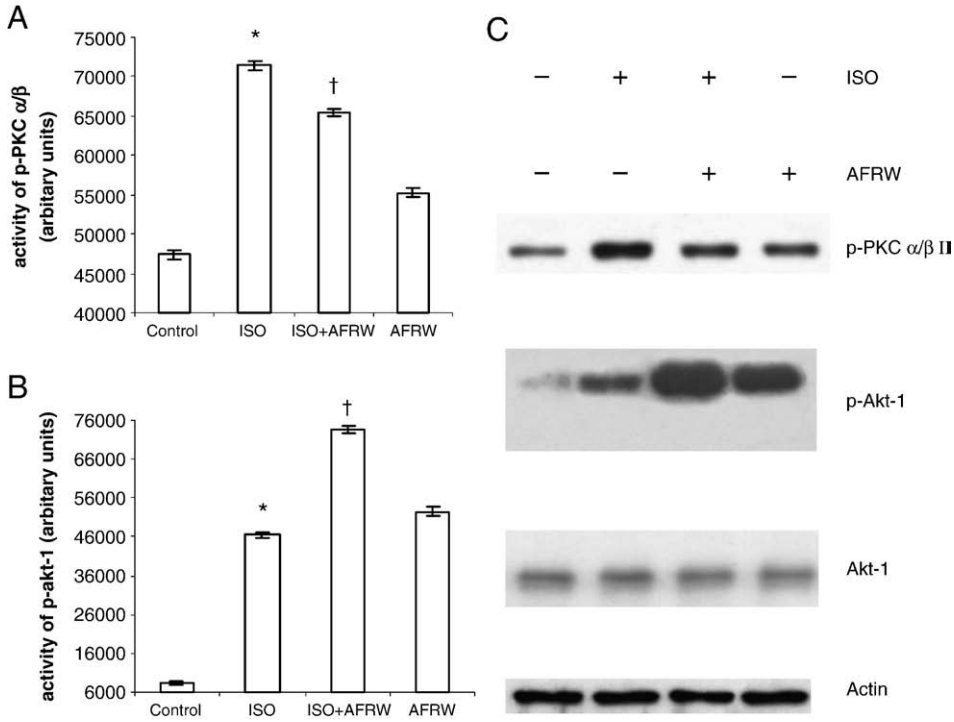

Fig. 4. Effect of AFRW and ISO on activation of PKC α/β II and Akt. Eight weeks after ISO-induced CHF, hearts were subjected to Western blot analysis using phosphorylation-specific primary antibodies against PKC α/β II (A,C) and Akt (B,C). Pixel densities of protein bands were determined by using the NIH Image J software and were expressed as mean±S.E.M. (n=5) (A,B). Representative immunoblots from five experiments with similar results are shown (C). Actin and total (phosphorylated and unphosphorylated) Akt were used as loading controls, and the results were normalized to the respective actin band. *Significantly different from control, $P<.05$. †Significantly different from ISO, $P<.05$.

growth that occurs in response to endothelin-1 stimulation or pressure overload [31], this cytoprotective pathway can have a crucial role and may represent a therapeutic target in postinfarction heart, too. In addition, Akt promotes cell survival by inhibiting apoptosis at multiple points, modulates carbohydrate metabolism, regulates protein synthesis, and phosphorylates and activates endothelial nitric oxide synthase which is important in the regulation of cardiac function and is present in both myocytes and cardiac endothelial cells. In the present study, we did not find any significant alterations in the phosphorylation of different MAPKs we studied (data not shown); however, we found increased phosphorylation of Akt in both ISO- and AFRW-treated hearts, and a much increased phosphorylation in ISO+AFRW-treated hearts.

Free-radical generation by the failing myocardium has been proposed as linked to myocardial remodeling [32] and was shown to induce Akt activation by oxidizing phosphatase and tensin homolog deleted from chromosome 10 (PTEN) [33], providing a defensive mechanism against the deleterious effects of the free radicals. Our findings that Akt was activated in the ISO group were in agreement with this widely accepted notion. We found that AFRW by itself induced Akt phosphorylation, and this phosphorylation was considerably enhanced in the ISO+AFRW-treated group. This pattern of activation of Akt was very similar to that found for PARP inhibitors in septic shock [34], oxidative stress [35] and ischemia-reperfusion [36] models for red wine polyphenols in an ischemia model [37] and for various cytoprotective agents in different stress-stimuli models.

Our results suggest that AFRW might be a new, complementary nutrient agent in patients with cardiovascular diseases, and it can prevent postinfarction cardiac remodeling by inhibiting PKC and activating Akt pathways.

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