

Upregulation of Genes Involved in Cardiac Metabolism Enhances Myocardial Resistance to Ischemia/Reperfusion in the Rat Heart

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Summary

Genes encoding enzymes involved in fatty acids (FA) and glucose oxidation are transcriptionally regulated by peroxisome proliferator-activated receptors (PPAR), members of the nuclear receptor superfamily. Under conditions associated with O₂ deficiency, PPAR- α modulates substrate switch (between FA and glucose) aimed at the adequate energy production to maintain basic cardiac function. Both, positive and negative effects of PPAR- α activation on myocardial ischemia/reperfusion (I/R) injury have been reported. Moreover, the role of PPAR-mediated metabolic shifts in cardioprotective mechanisms of preconditioning (PC) is relatively less investigated. We explored the effects of PPAR- α upregulation mimicking a delayed "second window" of PC on I/R injury in the rat heart and potential downstream mechanisms involved. Pretreatment of rats with PPAR- α agonist WY-14643 (WY, 1 mg/kg, i.p.) 24 h prior to I/R reduced post-ischemic stunning, arrhythmias and the extent of lethal injury (infarct size) and apoptosis (caspase-3 expression) in isolated hearts exposed to 30-min global ischemia and 2-h reperfusion. Protection was associated with remarkably increased expression of PPAR- α target genes promoting FA utilization (medium-chain acyl-CoA dehydrogenase, pyruvate dehydrogenase kinase-4 and carnitine palmitoyltransferase I) and reduced expression of glucose transporter GLUT-4 responsible for glucose transport and metabolism. In addition, enhanced Akt phosphorylation and protein levels of eNOS, in conjunction with blunting of cardioprotection by NOS inhibitor L-NAME, were observed in the WY-treated hearts. Conclusions: upregulation of PPAR- α target metabolic genes involved in FA oxidation may

underlie a delayed phase PC-like protection in the rat heart. Potential non-genomic effects of PPAR- α -mediated cardioprotection may involve activation of prosurvival PI3K/Akt pathway and its downstream targets such as eNOS and subsequently reduced apoptosis.

Key words

Myocardial ischemia • Apoptosis • PPAR activation • Metabolic genes • Delayed preconditioning • WY-14643

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Introduction

Myocardial ischemia causes alterations in the dynamic balance between fatty acids (FA) β (beta)-oxidation (FAO) and glucose oxidation as the main energy-producing pathways in the heart that may dramatically jeopardize restoration of cardiac function and survival during reperfusion known as ischemia/reperfusion (I/R) injury (Jaswal *et al.* 2011). Genes encoding enzymes of FA transport/uptake and utilization *via* mitochondrial FAO as the primary source of ATP in the adult healthy heart are regulated by ligand-activated peroxisome proliferator-activated receptors (PPAR), members of nuclear hormone receptors

superfamily (Desvergne and Wahli 1999). Isoforms of PPAR identified so far, α (alpha), β/δ (beta/delta) and γ (gamma), are expressed in many species including rodents (Kliwer *et al.* 2001) and humans (Greene *et al.* 1995) and regulate different sets of genes with different biological function (Feige *et al.* 2006). PPAR- α isoform has been recognized as the central regulator of mitochondrial FAO (Finck 2007). Moreover, under physiological and pathological conditions associated with acute or chronic oxygen deprivation, PPAR- α modulates myocardial substrate selection (FA versus glucose) aimed at the maintenance of adequate energy production to preserve basic cardiac function (Huss and Kelly 2004, Lopaschuk *et al.* 2010).

Nevertheless, whether PPAR activation plays a beneficial or deleterious role in the myocardial response to acute I/R still remains a matter of controversy. Some authors (Panagia *et al.* 2005, Sambandam *et al.* 2006) have documented a negative impact of PPAR- α overexpression on myocardial functional recovery upon I/R indicating that increased rates of FAO (at the expense of glucose oxidation) may be detrimental to the heart during I/R. In line, under conditions linked with limitations in oxygen supply, glucose as a fuel may be beneficial for the heart by decreasing oxygen consumption. Thus, interventions that increase glucose oxidation and suppress FAO appear to be beneficial for the postischemic recovery of the myocardium (Barger *et al.* 2000).

On the other hand, targeted deletion of PPAR- α resulted in a larger size of infarction in PPAR- $\alpha^{-/-}$ mice subjected to ischemic challenge (Yue *et al.* 2003). In acute settings of various *in vivo* and *ex vivo* models of I/R, a decrease of PPAR- α , in conjunction with the metabolic effects, was observed, while postischemic myocardial dysfunction and the size of infarction were attenuated by synthetic ligands of PPAR- α (Bulhak *et al.* 2009, Tian *et al.* 2006, Yeh *et al.* 2006, Yue *et al.* 2003). In our recent studies, we have demonstrated that 5-days treatment of rats with simvastatin or with PPAR- α agonist WY-14643 significantly increased both baseline and post-I/R PPAR- α mRNA levels which was linked with enhanced cardiac ischemic tolerance (Ravingerová *et al.* 2009, 2012). These studies do not support the view of the beneficial role of FAO inhibition in the mechanisms of protection, at least in the experimental setting of acute I/R.

Although the role of metabolic changes mediated by PPAR in the mechanisms of various forms

of preconditioning (PC) and long-lasting cardiac adaptation (Kolář *et al.* 2007, Matejíková *et al.* 2009a,b, Naderi *et al.* 2010) is relatively less known, it has been proposed that PPAR- α and PPAR- γ isoforms may be involved in the mechanisms of a delayed „second window“ of protection that occurs 24 h after the application of initial preconditioning stimulus (Lotz *et al.* 2011a) and “remote“ PC (Lotz *et al.* 2011b). However, it is still a matter of debate whether cardioprotection is attributable to the modulation of cardiac energy metabolism or to non-metabolic effects of PPAR- α , such as antioxidative and antiapoptotic effects (Nun *et al.* 2011, Smeets *et al.* 2007, Yeh *et al.* 2006).

In order to clarify this issue, a compound WY-14643 (WY) as one of the most potent selective PPAR- α agonists (Forman *et al.* 1997) was administered to the rats 24 h prior to ischemia in order to mimic a delayed “second window” of PC. We focused on the investigation of changes in the cardiac expression of PPAR- α target genes involved in FA oxidation, medium-chain acyl-CoA dehydrogenase (MCAD) and carnitine palmitoyl-transferase I (mCPT-1), and genes regulating glucose transport (GLUT-4) and metabolism (pyruvate dehydrogenase kinase-4, PDK-4) (Finck 2007, Patel and Korotchikina 2006). We also aimed to examine the involvement of potential non-genomic prosurvival mechanisms activated by PPAR- α , such as PI3K/Akt and its downstream targets including eNOS and caspase-3 as a marker of apoptosis (Moissac *et al.* 2000).

Materials and Methods

Animals

Totally 62 male Wistar rats (250-300 g body weight), fed a standard diet and tap water *ad libitum*, were employed, of which 44 animals were used in the physiological studies and 18 rats were used in the studies of gene and protein expression. The animals were kept under standard conditions with a constant 12:12 h light/dark cycle (lights on at 06.00 h) and temperature ($22\text{ }^{\circ}\text{C} \pm 2\text{ }^{\circ}\text{C}$). PPAR- α agonist WY was administered in a single dosage (1 mg/kg, i.p.) 24 h prior to induction of ischemia. Untreated controls were given a similar amount of a solvent (dimethyl sulfoxide, DMSO). All studies were performed in accordance with the Guide for the Care and Use of Laboratory Animals published by US National Institutes of Health (NIH publication No 85-23, revised 1996) and approved by the Animal Health and Animal Welfare Division of the State Veterinary and

Food Administration of the Slovak Republic.

Perfusion technique

Rats were anesthetized (sodium pentobarbitone, 60 mg/kg, i.p.) and given heparin (500 IU, i.p.). Hearts were rapidly excised, placed in ice-cold perfusion buffer, cannulated *via* the aorta and perfused in the Langendorff mode at a constant perfusion pressure of 70 mmHg and at 37 °C. The perfusion solution was a modified Krebs-Henseleit buffer gassed with 95 % O₂ and 5 % CO₂ (pH 7.4) and containing (in mmol/l): NaCl, 118.0; KCl, 3.2; MgSO₄, 1.2; NaHCO₃, 25.0; KH₂PO₄, 1.18; CaCl₂, 2.5; glucose, 7.0. The solution was filtered through a 5 µm filter (Millipore) to remove contaminants. An epicardial electrogram (EG) was registered by means of 2 electrodes attached to the apex of the heart and the aortic cannula.

Contractile function evaluation

Left ventricular (LV) pressure was measured by means of a non-elastic water-filled balloon inserted into the LV cavity (inflated to obtain end-diastolic pressure of 5-7 mmHg) and connected to a pressure transducer (MLP844, ADInstruments, Germany). LV systolic (LVSP) and end-diastolic pressure (LVDiP), LV developed pressure (LVDP; systolic minus diastolic pressure), maximal rates of pressure development and fall, $+dP/dt_{max}$ and $-dP/dt_{max}$, as the indexes of contraction and relaxation, heart rate (HR, derived from electrogram) and pressure-rate product (PRP; LVDP x HR) as an index of overall heart performance were evaluated.

The above parameters, as well as the coronary flow were monitored during stabilization and the pre-ischemic period (for the evaluation of the drug effects), and were continuously recorded until the end of experiment. Heart function and arrhythmias were analyzed using PowerLab/8SP Chart 7 software (ADInstruments, Germany). The hearts were allowed to stabilize (15 min) before further interventions. Recovery of function after 40 min of reperfusion was expressed as percentage of pre-ischemic baseline values.

Protocols of ischemia/reperfusion

All hearts of WY-treated and untreated animals were randomly assigned to the following protocols (n=10-12 per group):

1) In two control groups, after stabilization and additional 15-min perfusion, the hearts of WY-treated and untreated rats were exposed to global ischemia

induced by clamping of the aortic inflow for 30 min followed by 2-h reperfusion for the measurement of reperfusion-induced arrhythmias, contractile recovery and the size of myocardial infarction.

2) To elucidate the role of NO in myocardial response to ischemia and in WY-induced cardioprotection, after stabilization, the hearts of WY-treated and untreated rats were subjected to 15-min perfusion with the inhibitor of NO synthase L-omega-nitro-N-arginine methyl ester (L-NAME; 0.1 mmol/l) prior to ischemia followed by the same protocol as in 1.

Infarct size determination

The infarct size (IS) area was delineated by staining with 2,3,5-triphenyltetrazolium chloride and determined by a computerized planimetric method as described earlier (Ravingerová *et al.* 2007). The IS was expressed as a percentage of the area at risk (AR) size.

Quantification of arrhythmias

Ventricular arrhythmias were recorded continuously during early 10-min period of reperfusion when the heart is most vulnerable to reperfusion-induced arrhythmias and were analyzed according to the Guidelines for the study of ischemia- and reperfusion-induced arrhythmias known as the Lambeth Conventions (Walker *et al.* 1988). We focused on the ectopic activity expressed as a total number of premature ventricular complexes (PVC) and duration of ventricular tachycardia (VT), which was defined as a run of four or more consecutive ectopic beats.

Tissue sampling

For the determination of mRNA levels of GLUT-4, MCAD, mCPT-1, and PDK-4, as well as protein levels of Akt, phosphorylated Akt (p-Akt), eNOS, and caspase-3, left ventricular tissue samples were taken at baseline conditions (BL) and after 30-min ischemia followed by 40-min reperfusion (I/R) in additional subgroups following the same protocols (4-5 hearts per group were used for the investigation of gene expression and the same number of hearts was used for the studies of protein expression, totally 18 hearts). Samples were stored in liquid nitrogen until usage.

RNA preparation and quantitative PCR

The relative expression of endogenous GLUT-4, MCAD, mCPT-1 and PDK-4 mRNA was determined by reverse transcription (RT) of total RNA followed by Real

Table 1. Pre-ischemic hemodynamic parameters of the isolated hearts of untreated control rats and WY-14643 (WY)-treated animals. Effects of perfusion with the inhibitor of NO synthase L-omega-nitro-N-arginine methyl ester (L-NAME).

Parameters	Baseline		Pre-ischemia (after L-NAME)	
	Control n=10	WY-treated n=12	Control n=10	WY-treated n=12
HR (beats/min)	271 ± 15	287 ± 14	266 ± 16	294 ± 14
LVSP (mmHg)	93 ± 3.5	99 ± 5.6	101 ± 4.5	91 ± 5.6
LVDiP (mmHg)	5.5 ± 1.7	4.4 ± 0.6	5.0 ± 1.2	5.2 ± 0.6
LVDP (mmHg)	86 ± 3.2	95 ± 8.1	96.0 ± 7.6	84.0 ± 7.2
+(dP/dt) _{max} (mmHg/s)	2664 ± 150	2494 ± 180	2483 ± 100	2458 ± 170
-(dP/dt) _{max} (mmHg/s)	1970 ± 160	1785 ± 120	1743 ± 102	1697 ± 210
PRP (mmHg x beats/min)	26223 ± 1540	27120 ± 1670	25630 ± 1940	26132 ± 1052
CF (ml/min)	6.8 ± 0.3	6.4 ± 1.0	6.4 ± 0.4	6.3 ± 0.5

WY-14643 – agonist of PPAR- α ; HR – heart rate; LVSP – left ventricular systolic pressure; LVDiP – left ventricular end-diastolic pressure; LVDP – left ventricular developed pressure (LVSP minus LVDiP); \pm /(dP/dt)_{max} – maximal rates of pressure development and fall; PRP – pressure-rate product (LVDP x HR); CF – coronary flow. Values are means \pm S.E.M. from 10-12 hearts per group. No significant differences in the parameters investigated were found between the groups.

time PCR analysis. Total RNA was prepared using TRI reagent (Molecular Research Center) according to the manufacturer's protocol. The RNA samples were resuspended in 0.1 % (v/v) diethylpyrocarbonate (DEPC)-treated water and were quantified spectrophotometrically at 260 nm. RT was performed on 1 mg of total RNA using 100 IU of MMLV RTase (Finnzymes, Finland) in the presence of 100 ng random hexanucleotide primers (Takara BIO, Japan) in a 20 ml reaction volume. qPCR analysis was performed using a Real-time PCR system (Applied Biosystems). The sequences of the forward and reverse primers used for amplification were: Glut-4, 5' GGC TCT GAA GAT GGG GAA C 3' and 5' CGC GTT GAT GAC TCC AAT G 3'; MCAD, 5' CTT CGA GTT GAC GGA GCA G 3' and 5' TTG ATG AGA GGG AAC GGG T 3'; PDK-4, 5'-AGCTGCTGGACTTCGGTTCA-3' and 5'-GCGTTCAGGGAGGATGTCAA-3'; mCPT-1, 5' CTGGGCTTCTGTGTTCGTC 3' and 5' AATTGTGGCTGGCACA CTG 3'. Each reaction mix contained 12.5 ml SYBR FAST qPCR Master Mix (KAPA BIOSYSTEMS), 5 ml oligonucleotides (10 pmol each of forward and reverse primers), and 7.5 ml cDNA (diluted 1:10). qPCR analysis of β -actin was performed as an endogenous control with forward 5'-GCCCTGAGGCACTCTTCCA-3' and reverse 5'-GGATGTCCACGTCACACTTC-3' primers. After qPCR, dissociation curve analysis was routinely performed to check for aberrant amplification products

(e.g. primer-dimers).

Electrophoresis and Western blot analysis

Samples of protein fractions were separated by sodium dodecyl sulfate-polyacrylamide (10 %) gel electrophoresis (SDS-PAGE). For Western blot assays, after an electrophoretic separation, proteins were transferred to a nitrocellulose membrane. Antibodies against Akt (Santa Cruz Biotechnology), phospho-Akt(Ser473) (Cell Signaling Technology), eNOS (Cell Signaling Technology), caspase-3 (Santa Cruz Biotechnology) were used for the primary immunodetection. Peroxidase-labelled anti-rabbit immunoglobulin (Cell Signaling Technology) was used as the secondary antibody. Bound antibodies were detected by the enhanced chemiluminescence (ECL) method. The optical density of individual bands was analyzed by PCBAS 2.08e software and normalized to GAPDH as an internal control.

Chemicals

WY-14643 and L-NAME were obtained from Sigma (St. Louis, MO, USA). WY-14643 was dissolved in 10 % DMSO (Lachema, Czech Republic). All other chemicals were from Centralchem (Bratislava, Slovak Republic).

Statistical evaluation

The data were expressed as means \pm S.E.M.

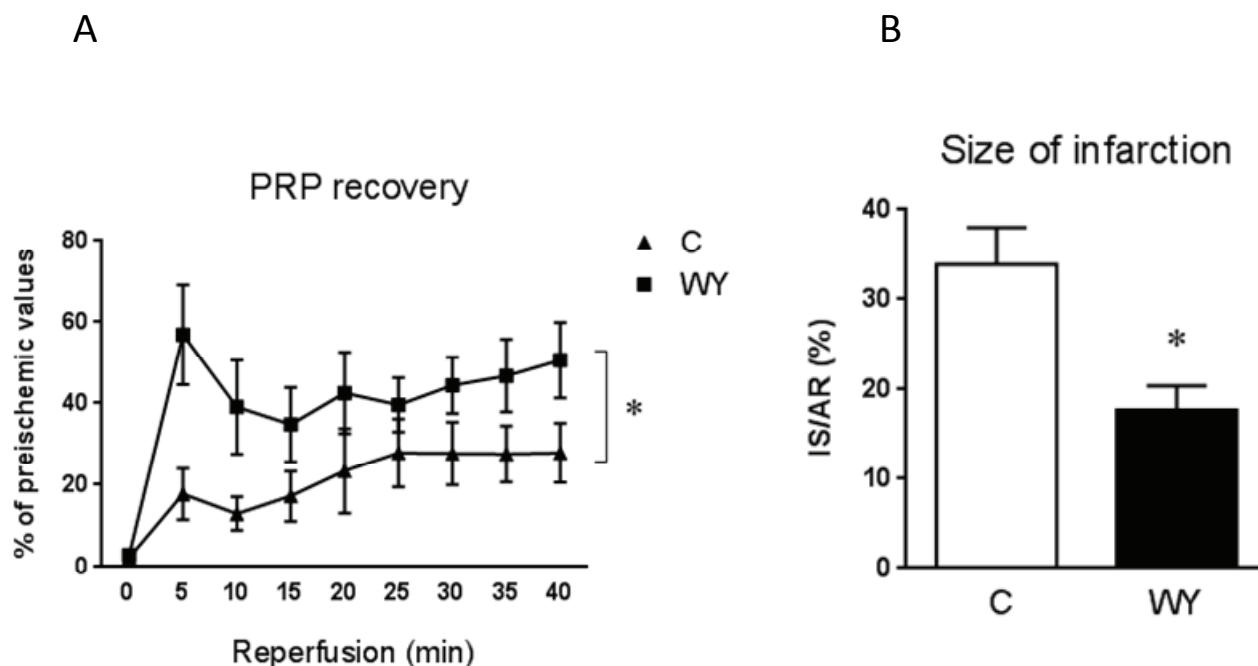


Fig. 1. Effect of 24-h pretreatment with WY-14643 (WY) on the ischemia-reperfusion (I/R) injury after 30-min global ischemia and 2-h reperfusion in the Langendorff-perfused rat hearts. **A.** Time-course of post-ischemic recovery of PRP (pressure-rate product) during 40-min reperfusion. **B.** Size of myocardial infarction (IS) expressed in % of area at risk (AR) size. Data are means \pm S.E.M. from 10-12 hearts per group. * $P < 0.05$ vs. untreated control group

One-way ANOVA and subsequent Student-Newman-Keuls test were used for comparison of differences in variables with normal distribution between the groups. Variables with non-parametric distribution (the number of PVC and duration of VT) were compared by using Mann-Whitney U test. Differences were considered as significant at $P < 0.05$.

Results

Characteristics of isolated hearts

No differences in the values of heart weight between the untreated and WY-treated groups were observed. The values of heart rate, LVSP, LVDiP, LVDP, $+dP/dt_{max}$, $-dP/dt_{max}$, PRP and coronary flow in the untreated control and WY-pretreated groups, as well as in L-NAME-treated control and WY-pretreated groups are summarized in Table 1. There were no significant differences in the values of these parameters between the groups at baseline and after administration of L-NAME prior to ischemia.

Effect of WY administration on postischemic recovery of contractile function, size of infarction and susceptibility to ventricular arrhythmias

Pretreatment of rats with WY resulted in a

delayed cardioprotection in the isolated hearts subjected to I/R 24 h later. Postischemic recovery of contractile function (PRP) in the WY-treated hearts was improved during the whole 40-min time-course of reperfusion as compared with the untreated controls (Fig. 1A). At the end of this period, LVDP was significantly increased in these hearts (61 ± 9 % of pre-ischemic values vs. 24 ± 3 % in the controls; $P < 0.05$; Fig. 2A).

The size of myocardial infarction was significantly reduced from 34 ± 4 % of the area at risk in the controls to 18 ± 3 % in the hearts from WY-treated rats ($P < 0.05$; Fig. 1B).

Reperfusion of the ischemic myocardium resulted in the high incidence of severe ventricular tachyarrhythmias. However, the total number of PVC observed in the WY-treated group was significantly smaller (Fig. 2B) and the duration of VT was significantly shorter (12.7 ± 5.3 s vs. 100 ± 26 s; $P < 0.05$; Fig. 2C).

NOS inhibition and protein levels of eNOS in WY-treated and untreated control hearts

Perfusion of the control hearts with NOS inhibitor L-NAME resulted in myocardial protection against postischemic contractile dysfunction (significantly improved recovery of LVDP; Fig. 2A), as

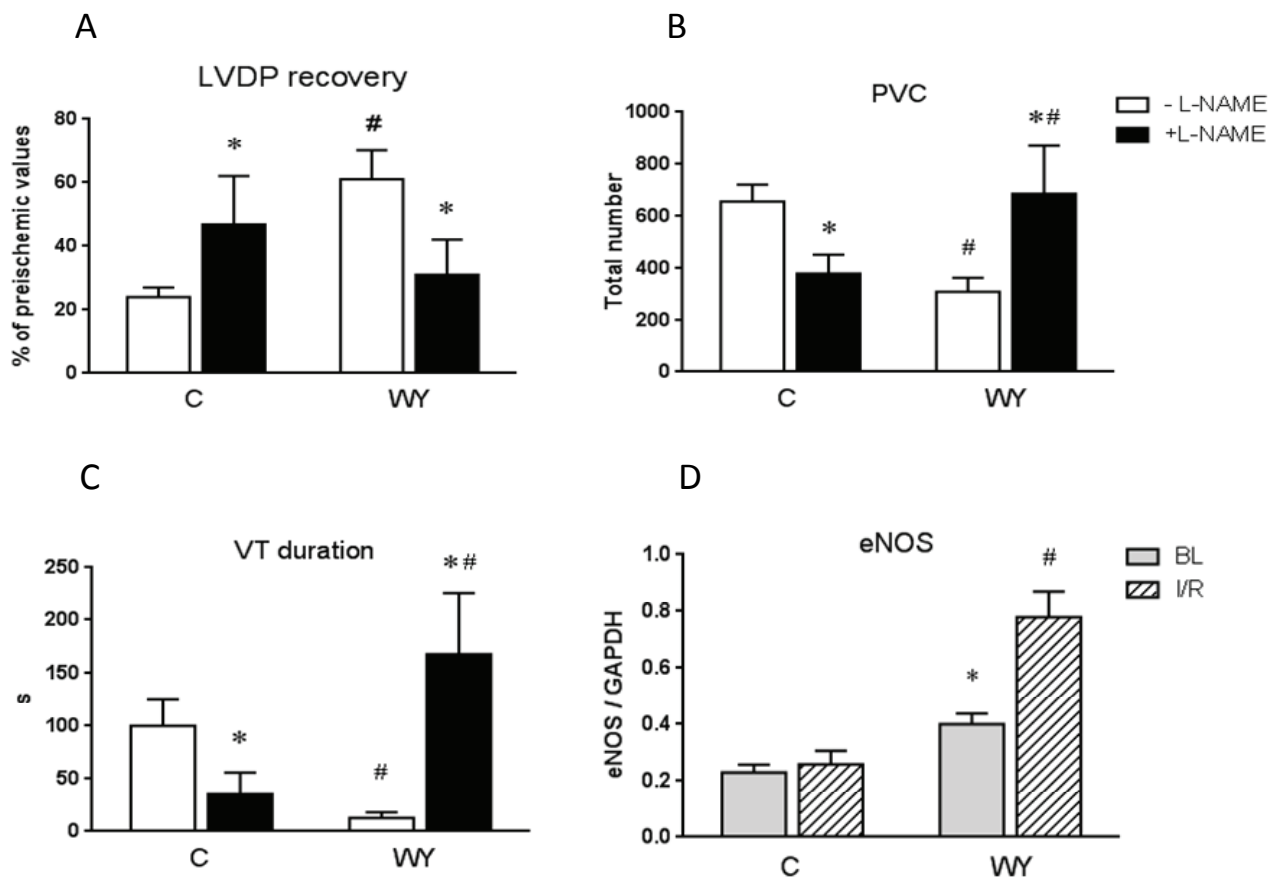


Fig. 2. Effect of 24-h pretreatment with WY-14643 (WY) on the ischemia-reperfusion (I/R) injury in the Langendorff-perfused rat hearts treated and untreated with NO synthase inhibitor L-NAME. **A.** Recovery of left ventricular developed pressure (LVDP) after 40-min reperfusion expressed in % of pre-ischemic values. **B.** Total number of premature ventricular complexes (PVC). **C.** Duration of ventricular tachycardia (VT, s). Data are means \pm S.E.M. from 10-12 hearts per group taken during 10-min reperfusion. * $P < 0.05$ vs. L-NAME-untreated groups. # $P < 0.05$ vs. WY-untreated controls (C). **D.** Protein levels of eNOS at baseline (BL) and after 30-min global ischemia followed by 40-min reperfusion (I/R) are presented as means \pm S.E.M. for 4 and 5 hearts in the untreated control and WY-treated group, respectively, normalized to the levels of GAPDH. * $P < 0.05$ relative to untreated controls at BL; # $P < 0.05$ relative to WY-treated controls at BL.

well as reduced ectopic activity (Fig. 2B) and duration of VT (Fig. 2C). On the contrary, in the WY-treated group, restoration of LVDP was reversed to its values in the untreated hearts (31 ± 11 %; $P > 0.05$ vs. controls; Fig. 2A) and the total number of PVC did not differ from that in the L-NAME-untreated control group (Fig. 2B). In addition, duration of VT was dramatically increased to 168 ± 58 s ($P < 0.05$ vs. WY-treated group without perfusion with L-NAME; Fig. 2C). Although L-NAME reduced the size of infarction in the controls by 47 %, in the WY-treated group it tended to blunt IS-limiting effect of WY (data not shown).

As shown in Fig. 2D, protein expression of eNOS was not modified by I/R in the myocardium of the control untreated rats. On the other hand, administration of PPAR- α agonist led to a significant increase in the protein levels of eNOS both, at baseline conditions and even more remarkable elevation of eNOS levels was

observed after I/R as compared with its post-I/R levels in the control untreated group.

Effect of PPAR- α activation on the expression of metabolic genes involved in the metabolism of fatty acids and glucose

Expression of mCPT-1 or MCAD mRNA was not significantly different at baseline between WY-treated animals and the untreated controls (Fig. 3A,B). However, after I/R, mRNA levels of mCPT-1 in the hearts of WY-treated animals were maintained at the baseline levels in contrast to their significant, more than 2-fold, decrease in the myocardium of the untreated controls (Fig. 3A). Similarly, post-I/R MCAD mRNA levels were significantly higher in WY-treated group than in the respective untreated controls, despite a tendency to decline as compared with the baseline levels (Fig. 3B).

Treatment with WY had no effect on the mRNA

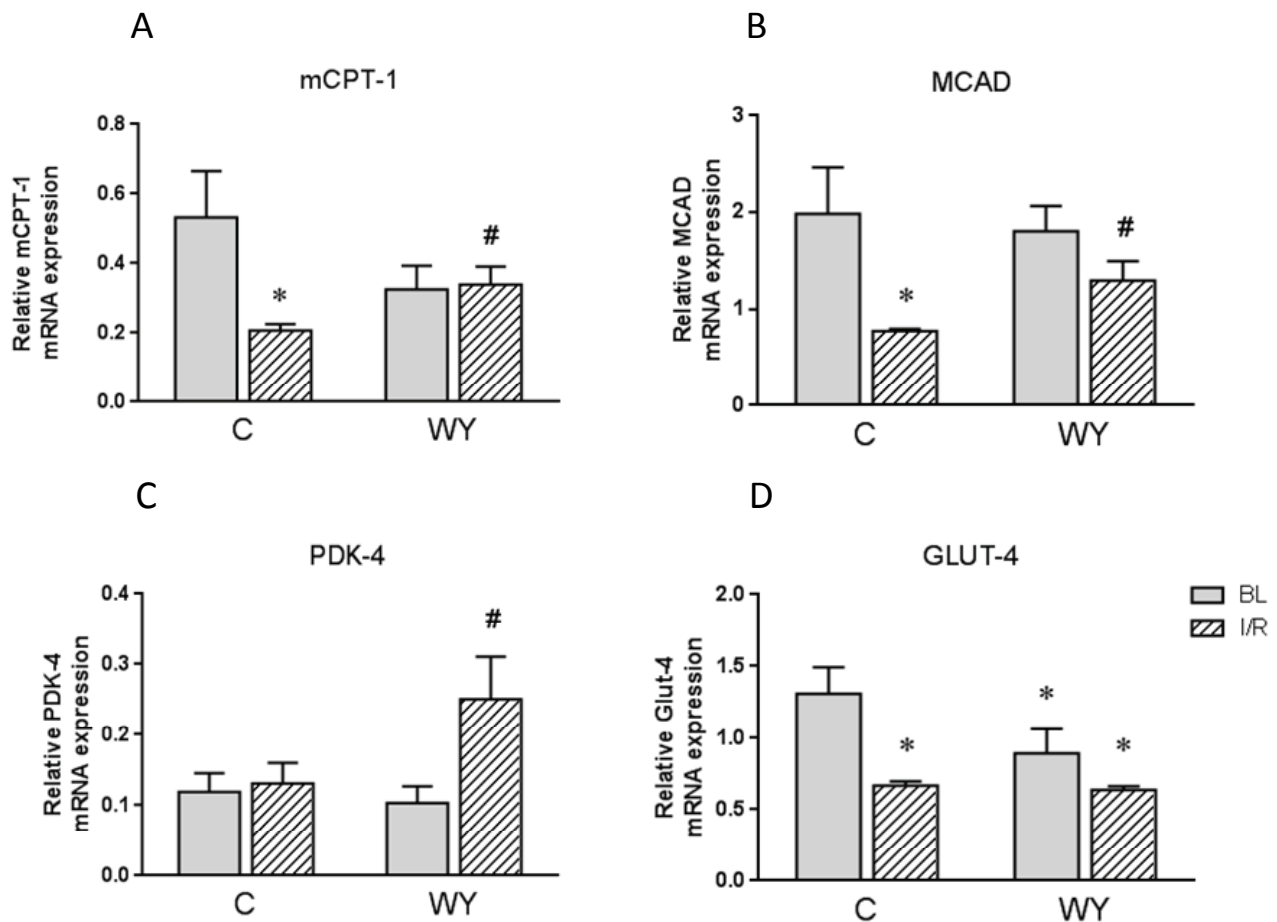


Fig. 3. Effect of pretreatment of rats with WY-14643 (WY) on gene expression of mCPT-1 (A), MCAD (B), PDK-4 (C) and GLUT-4 (D). Levels of mRNA in the hearts of control (C) and WY-treated animals (WY) at baseline (BL) and after 30-min global ischemia followed by 40-min reperfusion (I/R) were determined by real time RT-PCR. Levels of mRNA were normalized to the mRNA levels of the housekeeping gene GAPDH. The results are presented as means \pm S.E.M. for 4 and 5 hearts in the untreated control and WY-treated group, respectively. * $P < 0.05$ relative to untreated controls at BL; # $P < 0.05$ relative to WY-treated controls at BL.

levels of PDK-4 at baseline conditions (Fig. 3C). However, after I/R, mRNA levels of PDK-4 in the WY-treated group were markedly increased compared with the baseline values and with the post-I/R mRNA levels of PDK-4 in the untreated controls.

Baseline gene expression of GLUT-4 was significantly decreased in WY-treated group compared with the untreated controls (Fig. 3D). After I/R, mRNA levels of GLUT-4 in the untreated controls were markedly reduced compared with baseline levels. Post-I/R gene expression of GLUT-4 in WY-treated group was maintained at similar level compared with the untreated controls and, although there was a tendency to decrease compared with baseline in the treated group, the difference did not reach the level of significance.

Effect of PPAR- α activation on the phosphorylation of Akt before and after ischemia/reperfusion

Western blot analysis revealed no differences in

the protein levels of total Akt in any of the groups (Fig. 4A). However, the baseline preischemic levels of phosphorylated (activated) Akt were significantly increased 24 h after administration of WY in comparison with its levels in the untreated group. Enhanced phosphorylation of Akt was preserved during I/R in this group and was significantly higher than post-I/R levels of phospho-Akt in the untreated controls (Fig. 4A,B).

Effect of PPAR- α agonist on caspase-3 activation before and after ischemia/reperfusion

Caspase-3 activation was assessed by determining protein levels of inactive procaspase-3 and cleaved (activated) caspase-3 by Western blotting. A specific antibody against the full-length caspase-3 (35 kDa) and the large fragment (17 kDa) of activated caspase-3 resulting from cleavage was used. As shown in Fig. 4C and D, the levels of activated form of caspase-3 were moderately increased in response to I/R in the

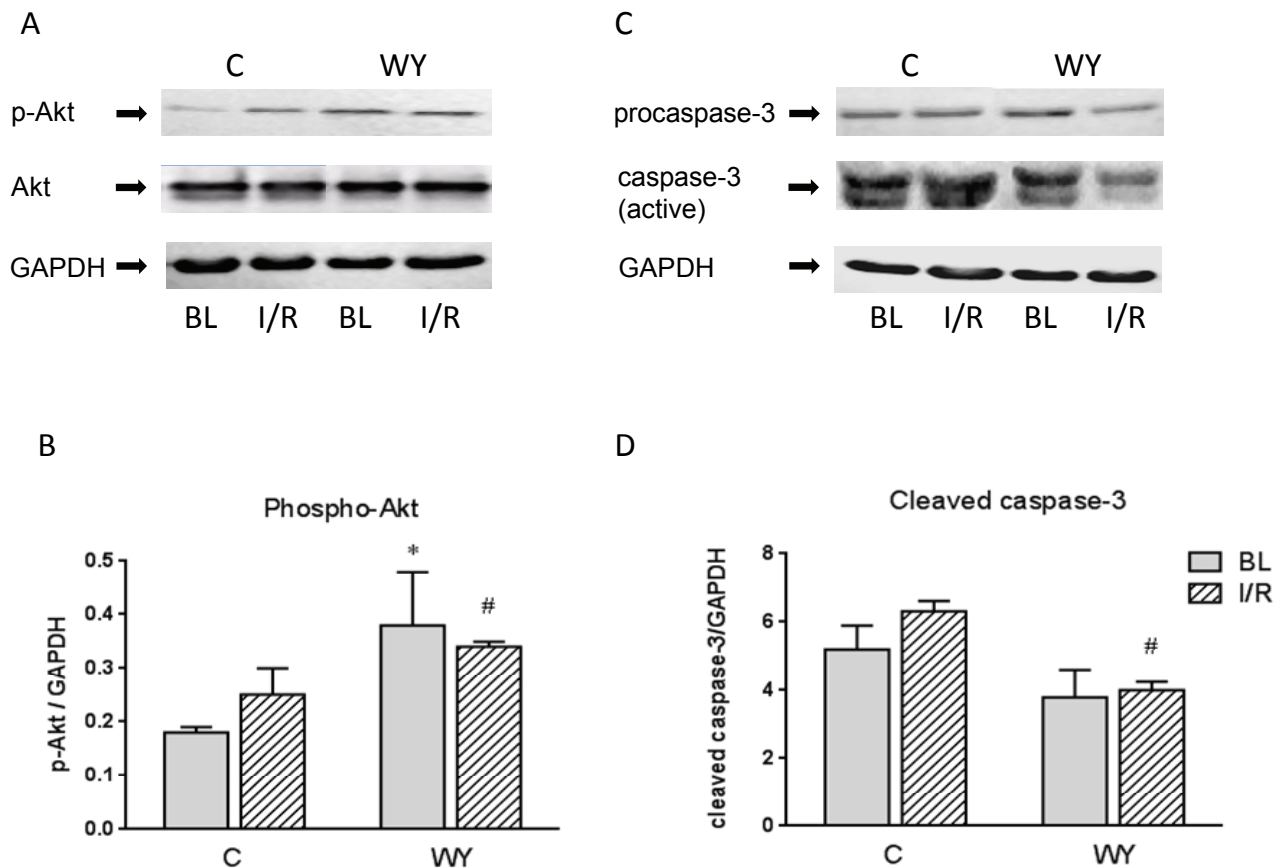


Fig. 4. Levels of phosphorylated Akt (**A, B**) and cleaved caspase-3 (**C, D**) in the hearts of control (C) and WY-treated animals (WY) at baseline (BL) and after 30-min global ischemia followed by 40-min reperfusion (I/R). **A.** Representative blots of total Akt and phosphorylated Akt (p-Akt). **C.** Representative blots of procaspase-3 and cleaved (activated) caspase-3. Protein levels were normalized to the levels of GAPDH. The results are presented as means \pm S.E.M. for 4 and 5 hearts in the untreated control and WY-treated group, respectively. * $P < 0.05$ relative to untreated controls at BL; # $P < 0.05$ relative to WY-treated controls at BL.

myocardial tissue of control untreated animals. Importantly, reduced severity of I/R injury in the WY-treated group was associated with significantly lower levels of cleaved caspase-3 than in the ischemic tissue of the untreated control hearts (Fig. 4C,D).

Discussion

The role of PPAR- α in myocardial response to ischemia is still controversial, and although altered metabolism is a major target for cardioprotection, metabolic changes involved in the mechanisms of therapeutic interventions related to PPAR- α upregulation are relatively less clear. Declined cardiac gene expression of PPAR- α resulting in a substrate switch from FA to glucose is considered as an adaptive mechanism during chronic hypoxia (Razeghi *et al.* 2001), and it appears that increased rates of FAO may be detrimental to the heart in the chronic processes, due to enhanced oxygen consumption and due to FAO-induced oxidative stress as

well (Sambandam *et al.* 2006). However, activation of PPAR- α by its exogenous ligands (Bulhak *et al.* 2009, Wayman *et al.* 2002) resulted in the effective protection of the heart against I/R injury.

Thus, a primary goal of the study was to clarify whether the changes in the expression of the PPAR- α target metabolic genes may underlie cardioprotective effects in a setting of acute I/R. In our previous study, WY caused a two-fold increase in PPAR- α mRNA levels in the rat myocardium after 5-days (3 mg/kg, p.o.) application (Ravingerová *et al.* 2012) accompanied by the protection of the heart against global I/R. On the other hand, this compound has been already shown to afford an effective cardioprotection in rats even when administered shortly (30 min) prior to ischemia in the same concentration as in our study (Bulhak *et al.* 2009). Similar to other studies demonstrating cardioprotective effects of synthetic PPAR- α ligands in a setting of acute I/R (Wayman *et al.* 2002, Yue *et al.* 2003, Tian *et al.* 2006), we have observed a protective effect of

pretreatment of rats with WY on the postischemic contractile dysfunction (Fig. 1A, 2A), size of infarction (Fig. 1B) and severity of reperfusion-induced arrhythmias (Fig. 2B,C).

Effect of PPAR- α activation on the expression of metabolic genes

In the aforementioned studies, cardioprotection due to pretreatment of animals with different PPAR- α agonists prior to ischemia reversed down-regulation of PPAR- α and its target genes responsible for the metabolic fuel shifts (from decreased FAO to increased glucose oxidation) (Yue *et al.* 2003). In agreement with these studies, our study revealed that pretreatment of rats with PPAR- α agonist WY resulted in post-I/R preservation of mRNA levels of mCPT-1 in the hearts of WY-treated animals, in contrast to their significant decline compared to the baseline levels in the myocardium of the untreated controls (Fig. 3A). Similar, post-I/R MCAD mRNA levels were significantly higher in WY-treated group than in the respective untreated controls (Fig. 3B). These findings indicate a higher reliance on FA as a source of energy in the ischemic myocardium of animals treated with PPAR- α agonist. Furthermore, although WY did not modify mRNA levels of PDK-4 at baseline conditions (Fig. 3C), postischemic PDK-4 mRNA levels were markedly increased in the WY-treated group compared with the baseline and with the post-I/R mRNA levels of PDK-4 in the untreated controls. As a target metabolic gene of PPAR- α , PDK-4 can phosphorylate (and inactivate) pyruvate dehydrogenase complex (PDC) as a major regulator of glucose and lactate oxidation and can block the flow of glycolytic intermediates into the tricarboxylic acid cycle (Song *et al.* 2010). In line, baseline gene expression of glucose transporter GLUT-4 was significantly decreased in WY-treated group compared with the untreated controls (Fig. 3D). Previous studies have also shown that cardiac-specific overexpression of PPAR- α resulted in repression of the expression of GLUT-4 gene, while treatment of these mice with fenofibrate, a PPAR- α agonist, led to a further decrease in GLUT-4 mRNA levels (Burkart *et al.* 2007). In our study, post-I/R mRNA levels of GLUT-4 in the untreated controls were markedly reduced compared with the baseline levels. On the other hand, post-I/R gene expression of GLUT-4 in WY-treated group was maintained at a similar level as in the untreated controls, although there was a tendency to decrease compared with the baseline levels in this group. Taken together, these

findings further support an important role of increased FAO, rather than glucose oxidation, as a mechanism required for the fast restoration of heart function under conditions of reperfusion of acutely ischemic myocardium, when oxygen supply is no more limited.

PPAR and preconditioning protection against ischemia/reperfusion

Although molecular mechanisms of endogenous cardioprotection have been extensively studied, metabolic changes involved in the mechanisms of PC and the role of PPAR are relatively less investigated. However, it has been reported that PPAR- γ activation prior to I/R by PPAR- γ agonist pioglitazone may mimic PC in the isolated perfused rat heart *via* activation of pro-survival kinases PI3K and P42/44MAPK (Wynne *et al.* 2005). Interestingly, PPAR- γ isoform has been reported to be involved in the mechanisms of a „second window“ of anaesthetic-induced PC (Lotz *et al.* 2011b) and both isoforms mediated „remote“ (renal ischemia-induced) PC against myocardial infarction in rabbits *in vivo* (Lotz *et al.* 2011a).

Thus, our further goal was to find out whether a single administration of exogenous PPAR- α agonist could serve as a mimetic of a delayed phase of cardioprotection, similarly to the “second window” of PC, and to identify potential non-metabolic cardioprotective mechanisms related to PPAR- α activation. To reproduce the protocol of a delayed PC, we applied a single dosage of PPAR- α agonist WY 24 h prior to I/R. Our findings support the view that the ligand-induced upregulation of PPAR- α in the heart prior to I/R may account for a better recovery of function (Fig. 1A, 2A), reduced size of infarction (Fig. 1B) and severity of reperfusion-induced arrhythmias (Fig. 2B,C), similar to dealed phase PC.

Effect of PPAR- α agonist on non-metabolic protective pathways

A cascade of PI3K/Akt involved in anti-apoptotic, anti-oxidative processes and in metabolic regulations (Murphy and Steenbergen 2007) appears to play a key role in the mechanisms of preconditioning protection against lethal injury and malignant arrhythmias (Mocanu *et al.* 2002, Ravingerová *et al.* 2007). Our recent study demonstrated that Akt phosphorylation was enhanced in the ischemic myocardium of PPAR- α agonist WY-treated rats, and protection against I/R conferred by 5-days administration of WY was abrogated by PI3K inhibitor wortmannin (Ravingerová *et al.* 2012).

Similarly, Bulhak *et al.* (2009) found increased levels and phosphorylation of Akt in the ischemic myocardium of Wistar rats already 30 min after pretreatment with WY, as well as the abrogation of IS-limiting effect of WY by wortmannin. In accord, in the present study, delayed cardioprotective effects of WY were associated with enhanced phosphorylation (activation) of Akt already at baseline that was preserved during I/R in this group and was significantly higher than post-I/R levels of phospho-Akt in the untreated controls (Fig. 4A,B).

NO synthase is considered as a downstream target of PI3K/Akt (Murphy and Steenbergen 2007) and its role in the „second window” PC and in PPAR- α -induced cardioprotection is well documented (Bolli *et al.* 1997, Wayman *et al.* 2002). In accordance with our previous study (Andelová *et al.* 2005) demonstrating a dual role of NO in the ischemic myocardium, the present study confirmed the negative role of NO and protective effect of NOS inhibition in the control untreated hearts exposed to I/R, as well as a positive role of NO in a setting of delayed cardioprotection induced by PPAR- α activation (Fig. 2A,B,C). Importantly, cardioprotective effects in the WY-treated group were associated with enhanced protein levels of eNOS already before ischemia and even more remarkable increase in eNOS levels after I/R (Fig. 2D) indicating a potential link between NO availability and delayed cardioprotection in WY-treated animals.

A major finding of the present study is that administration of PPAR- α agonist significantly reduced caspase-3 protein levels during subsequent I/R evoked in the rat hearts 24 h later (Fig. 4C,D). Caspases are cysteine proteinases that have been shown to be specifically involved in the initiation and execution phases of apoptosis (Schulz *et al.* 1999, Zeuner *et al.* 1999). Caspase-3 is detected in cardiomyocytes, and in some systems, Bcl-2 protein cleavage by activated caspase-3 promotes the release of cytochrome c leading to further cellular damage (Kirsch *et al.* 1999, Moissac *et al.* 2000). However, little has been reported regarding the mechanism of caspase-3 inhibition in the prevention of I/R injury, and the available evidence of the role of caspase-3 inhibition in a reduction of myocardial infarct size is controversial. While Yaoita *et al.* (1998) reported attenuation of I/R injury in rats by a caspase inhibitor, which could be at least partially attributed to the attenuation of cardiomyocyte apoptosis, in a study of Okamura *et al.* (2000), caspase activation was inhibited

by caspase inhibitors without reduction of the infarct size in ischemia-reperfused rat hearts. The results of our study show that cleavage (activation) of caspase-3 is suppressed by PPAR- α activation that might suggest a link between amelioration of I/R injury and activation of antiapoptotic processes, such as upregulation of Bcl-2 protein, the production and function of which may be modulated *via* alteration of caspase activity (Grünenfelder *et al.* 2001). However, we cannot exclude that other proteins involved in the PI3K/Akt-dependent mitochondrial apoptotic pathway (Murphy and Steenbergen 2007) might also contribute to the observed cardioprotective effects of PPAR- α activation.

Conclusions

Our results indicate that pretreatment of rats with the selective PPAR- α agonist WY-14643 confers an effective delayed protection against postischemic contractile dysfunction, severe ventricular arrhythmias and lethal injury in the hearts exposed to acute I/R 24 h later, similar to a second phase of PC. Cardioprotection in the hearts of WY-treated rats is associated with upregulation of PPAR- α target metabolic genes responsible for the higher utilization of FA under conditions of I/R. This is in contrast to the lower ischemic tolerance in the hearts of the untreated animals. The latter points out to an important role of an increased FAO in a setting of acute I/R due to the need to maintain adequate energy production for the fast restoration of heart function under conditions of resumed coronary flow, when oxygen supply is no longer rate limiting. Potential non-genomic mechanisms of cardioprotection induced by PPAR- α upregulation might be related to the activation of pro-survival cascades, including PI3K/Akt and its downstream eNOS, which leads to subsequent attenuation of the apoptotic processes in the myocardium.

Conflict of Interest

There is no conflict of interest.

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