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RAPID COMMUNICATION

Treatment of Rats With Hypolipidemic Compound Pirinixic Acid Protects Their Hearts Against Ischemic Injury: Are Mitochondrial K_{ATP} Channels and Reactive Oxygen Species Involved?

M. NEMČEKOVÁ[1], S. ČARNICKÁ[1], M. FERKO[1], M. MURÁRIKOVÁ[1], V. LEDVÉNYIOVÁ[1], T. RAVINGEROVÁ[1]

[1]Institute for Heart Research, Slovak Academy of Sciences, Centre of Excellence of SAS NOREG, Bratislava, Slovak Republic

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Summary

Hypolipidemic compound pirinixic acid (WY-14643, WY) is known to exert pleiotropic (other than primary) effects, such as activation of peroxisome proliferator-activated receptors (PPAR-α), transcription factors regulating different cardiac functions. Their role in ischemia-reperfusion (I/R) injury and cardioprotection is less clear, although protective effects of PPAR agonists have been documented. This study was designed to explore the effects of WY on the I/R injury in the rat heart and potential mechanisms involved, including mitochondrial K_{ATP} channels (mitoK_{ATP}) opening and production of reactive oxygen species (ROS). Langendorff-perfused hearts of rats intragastrally treated with WY (3 mg/kg/day) for 5 days and of control animals were subjected to 30-min global ischemia and 2-h reperfusion with or without 15-min perfusion with mitoK_{ATP} blocker 5-hydroxydecanoate (5-HD) prior to I/R. Evaluation of the infarct size (IS, TTC staining) served as the main end-point of protection. Lipid peroxidation (a marker of ROS production) was determined by measurement of myocardial concentration of conjugated dienes (CD), whereas protein expression of endothelial NO synthase was analysed by Western blotting. A 2-fold increase in the cardiac protein levels of eNOS after treatment with WY was accompanied by lower post-I/R levels of CD compared with those in the hearts of untreated controls, although WY itself enhanced ROS generation prior to ischemia. IS was reduced by 47 % in the hearts of WY-treated rats ($P<0.05$), and this effect was reversed by 5-HD. Results suggest that PPAR-α activation may confer protection against lethal I/R injury in the rat heart that involves up-regulation of eNOS, mitoK_{ATP} opening and reduced oxidative stress during I/R.

Key words

Myocardial ischemia-reperfusion injury • Cardioprotection • PPAR-α activation • Mitochondrial K_{ATP} channels • Reactive oxygen species • Endothelial NO synthase

Corresponding author

T. Ravingerová, Institute for Heart Research, Slovak Academy of Sciences, POB 104, Dúbravská cesta 9, 840 05 Bratislava, Slovak Republic. Fax: 421 2 5477 6637. E-mail: usrdravi@savba.sk

Pirinixic acid (WY-14643, WY) has been discovered as a hypolipidemic compound (Satilli *et al.* 1974) that has been recently considered as potentially useful for prevention of severe cardiac dysfunction, cardiomyopathy and heart failure as a result of lipid accumulation within cardiomyocytes (Wölkart *et al.* 2012). On the other hand, its pleiotropic (other than primary) non-lipid effects have been shown to be attributed to its property as a potent and selective agonist of peroxisome proliferator-activated receptors (PPAR), namely their PPAR-α isoform (Forman *et al.* 1997). The role of these nuclear receptors in myocardial ischemia/reperfusion (I/R) injury is still a matter of debate and some studies point out to the detrimental effects of PPAR-α activation (Sambandam *et al.* 2006).

However, while implication of PPAR-α in the mechanisms of innate cardioprotection remains less investigated, protective effects of PPAR synthetic agonists against I/R injury in non-diseased (Yue *et al.* 2003) and diabetic (Bulhak *et al.* 2009) heart have been reported. We and others have previously demonstrated that down-regulation of PPAR-α may be deleterious for the heart under conditions of I/R (Yue *et al.* 2003, Ravingerová *et al.* 2009). Moreover, cardioprotective effects of hypolipidemic drugs, such as statins (Ravingerová *et al.* 2009), and PPAR-α synthetic ligands fibrates (Sugga *et al.* 2012) or WY (Bulhak *et al.* 2009) appeared to be far beyond their lipid-lowering action and were associated with an increased gene (and protein) expression of PPAR-α and its target metabolic genes promoting fatty acids oxidation as a source of energy under conditions of acute I/R (Yue *et al.* 2003, Ravingerová *et al.* 2012). On the other hand, non-metabolic mechanisms of PPAR-α-induced cardioprotection are not completely clear, although it has been suggested that PPAR-α may be involved in the mechanisms of remote preconditioning (Lotz *et al.* 2011). The present study aimed to clarify potential downstream mechanisms of PPAR-α activation by its exogenous ligand taking into consideration the similarity with cardioprotection conferred by ischemic preconditioning (IPC): involvement of endothelial NO synthase (eNOS), mitochondrial K_{ATP} opening, and production of reactive oxygen species (ROS).

Male Wistar rats (250-300 g body weight), fed a standard diet and tap water *ad libitum*, were employed. Pirinixic acid (WY-14643, Sigma, St. Louis, USA) was given for 5 days at the dose of 3 mg/kg/day dissolved in 10 % dimethyl sulfoxide (DMSO), by gavage needle (Kent Scientific Corporation, USA). Control rats were given a similar amount of solvent (1.0 ml of 10 % 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.

After anesthesia (sodium pentobarbitone, 60 mg/kg, i.p.), hearts of WY-14643 (WY)-treated and DMSO-treated control rats were rapidly excised and perfused at 37 °C in the Langendorff mode. Krebs-Henseleit perfusion buffer gassed with 95 % O_2 and 5 % CO_2 contained (in mM): NaCl, 118.0; KCl, 3.2; $MgSO_4$, 1.2; $NaHCO_3$, 25.0; $KH2PO_4$, 1.18; $CaCl_2$ 2.5; glucose, 7.0. An epicardial electrogram was registered by means of two electrodes attached to the apex of the heart and the aortic cannula. Left ventricular (LV) pressure was measured by means of a non-elastic water-filled balloon inserted into the LV cavity. LV developed pressure (LVDP, systolic minus diastolic LV pressure), rates of pressure development and fall, heart rate and coronary flow were measured during pre-ischemic period for the evaluation of the potential effects of 5-hydroxydecanoate (5-HD, Sigma, St. Louis, USA) applied in perfusion buffer (200 μM) 15 min prior to the onset of ischemia and analysed until the end of experiments using PowerLab/8SP Chart 7 software (ADInstruments). Recovery of LVDP after ischemia and 40 min of reperfusion was expressed in % of pre-ischemic values.

For the evaluation of the infarct size (IS), the hearts of all groups were exposed to 30-min global ischemia followed by 2-h reperfusion. The area of infarct was determined by staining with 2,3,5-triphenyltetrazolium chloride and a computerized planimetric method as described earlier (Ravingerová *et al.* 2009). The IS was normalized to the size of area at risk (AR).

In parallel subsets of experiments, the sampling for conjugated dienes (CD, n=5-6 per group), a marker of lipid peroxidation due to increased ROS production was performed immediately after equilibration of the hearts (baseline, BL) and after ischemia followed by 40 min of reperfusion (I/R). Concentration of CD was measured in lipid extracts of the left ventricular tissue according to Kogure *et al.* (1982) adjusted to achieve optimal conditions for extraction of membrane lipids (Ziegelhöffer *et al.* 2009). Briefly, after chloroform evaporation under the inert atmosphere of nitrogen and after the addition of 3 ml cyclohexane, concentration of CD was determined spectrophotometrically (λ=223 nm, ε=29000 $l.mol^{-1}.cm^{-1}$, SECOMAN).

Left ventricular tissue samples from additional groups of the WY-treated and untreated animals (n=4-5 per group) were taken before ischemia for Western blot assays. Separation of protein fractions by sodium dodecyl sulfate-polyacrylamide (10 %) gel electrophoresis (SDS-PAGE) was followed by Western blot analysis. Anti-eNOS antibodies (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)

Table 1. Pre-ischemic hemodynamic parameters of the isolated hearts of untreated control rats and WY-14643 (WY)-treated animals. Effects of mitochondrial K_{ATP} channels blocker 5-hydroxydecanoate (5-HD).

	Baseline		Pre-ischemia (after 5-HD)	
Parameters	**Control**	**WY-treated**	**Control**	**WY-treated**
HR (beats/min)	237 ± 45	289 ± 21	248 ± 25	255 ± 7
LVSP (mm Hg)	92 ± 18	91 ± 7	97 ± 20	102 ± 20
LVDiP (mm Hg)	3.3 ± 0.3	4.7 ± 1.0	8.5 ± 2.6	3.7 ± 1.1
LVDP (mm Hg)	89 ± 18	86 ± 6	96 ± 17	98 ± 19
$+(dP/dt)_{max}$ *(mm Hg/s)*	2651 ± 575	3187 ± 319	2975 ± 437	3126 ± 664
$-(dP/dt)_{max}$ *(mm Hg/s)*	1678 ± 320	1638 ± 163	1995 ± 298	1985 ± 493
CF (ml/min)	7.7 ± 2.2	5.6 ± 0.4	7.3 ± 0.9	5.6 ± 1.1

WY-14643 – pirinixic acid, 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); $+/-(dP/dt)_{max}$ – maximal rates of pressure development and fall; CF – coronary flow. Values are means ± SEM from 8-10 hearts per group.

method. The optical density of individual bands was analyzed by PCBAS 2.08e software and normalized to GAPDH as an internal control.

The data were expressed as means ± SEM. One-way ANOVA and subsequent Student-Newman-Keuls test were used for comparison of differences in variables between the groups. Differences were considered as significant at $P<0.05$.

No significant differences in the values of coronary flow and hemodynamic parameters between WY-treated and untreated control groups were observed at baseline (Table 1). In addition, administration of 5-HD did not cause appreciable changes in these parameters in any of the groups.

Western blot analysis revealed that 5-days administration of PPAR-α agonist resulted in a significant, nearly 2-fold increase in protein expression of eNOS in left ventricular tissue of the hearts from the treated rats as compared with eNOS protein levels in the untreated control group at baseline conditions prior to ischemia (Fig. 1A).

In the group of hearts from WY-treated rats subjected to I/R, IS was significantly reduced from 34.0±4.0 % of AR to 18.0±3.0 % ($P<0.05$). Administration of 5-HD did not affect IS in the untreated control group (Fig. 1B). In contrast, in the WY-treated group, blockade of mitoK_{ATP} channels abrogated cardioprotective effect of WY and increased IS/AR to 28.0±4.0 % ($P<0.05$ as compared with the WY group without 5-HD; Fig. 1B).

Treatment of rats with WY resulted in a significantly better postischemic functional recovery (LVDP) from 24.0±3.0 % in controls to 61.0±9.0 % ($P<0.05$). Application of 5-HD did not influence LVDP recovery in the control group, however, it reversed protective effect of WY against contractile dysfunction to the level in the untreated controls (LVDP 35.0±8.0 %; $P>0.05$ vs. controls (Fig. 1C).

As expected, myocardial ischemia significantly increased lipid peroxidation and accordingly, concentration of CD was significantly higher in the postischemic myocardium of the untreated control animals (Fig. 1D). In contrast, although baseline CD levels in WY-treated hearts were higher than in the untreated ones, I/R did not enhance oxidative stress in these hearts, and postischemic CD levels were reduced as compared with their baseline levels in this group (Fig. 1D).

Hypolipidemic drugs (statins or fibrates) have been shown to be cardioprotective in a setting of I/R beyond their lipid-lowering effects as manifested by a reduced size of infarction and improved postischemic recovery of contractile function in different *in vivo* and *ex vivo* models in normocholesterolemic animals (Yue *et al.* 2003, Efthymiou *et al.* 2005, Ravingerová *et al.* 2009), that was confirmed in the present study (Fig. 1B,C). It has been proposed that PC-like pleiotropic effects of statins, as well as of fibrates, also known as PPAR-α ligands, are attributed to up-regulation of pro-survival pathways, such as phosphatidylinositol 3-kinase (PI3K)/Akt, ERK1/2 and eNOS (Bell and Yellon 2003, Wolfrum *et al.* 2004, Efthymiou *et al.* 2005, Merla *et al.* 2007, Bulhak *et al.* 2009). However, although inhibition of PPAR-α was able to abolish cardioprotective effects of

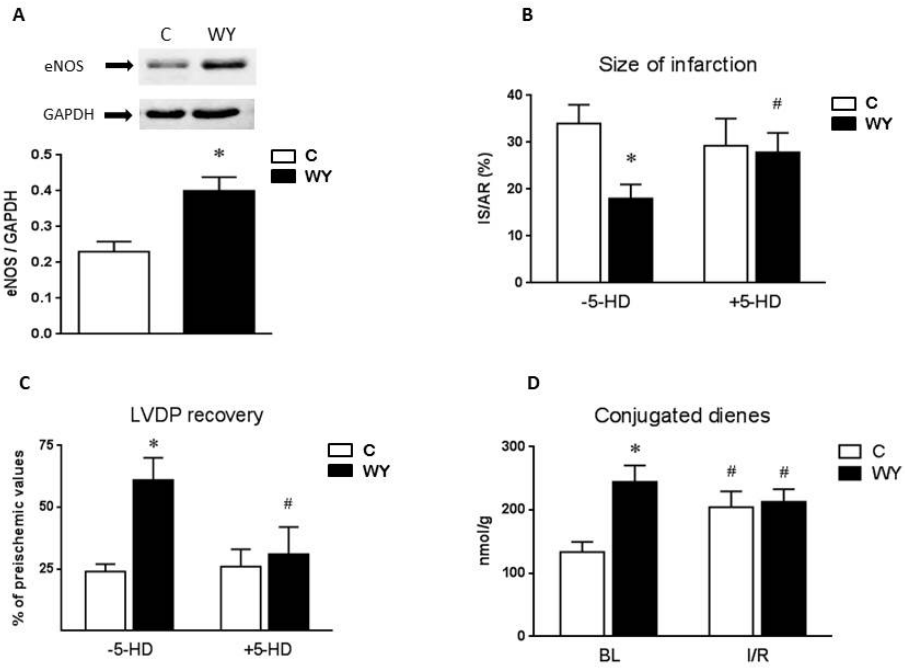

Fig. 1. Effect of 5-days treatment with PPAR-α agonist WY-14643 (WY) on the expression of endothelial NO synthase (eNOS) and ischemia-reperfusion (I/R) injury induced by 30-min global ischemia and 2-h reperfusion in Langendorff-perfused rat hearts. **A.** Protein levels of eNOS presented as means ± SEM for 4-5 hearts per group normalized to the levels of GAPDH. * P<0.05 relative to untreated controls (C). Representative immunoblot is shown on upper panel. **B.** Size of myocardial infarction (IS) in WY-treated and untreated hearts exposed to mitochondrial K_{ATP} blocker 5-hydroxydecanoate (5-HD). IS is expressed in % of area at risk (AR) size. **C.** Postischemic recovery of left ventricular developed pressure (LVDP, in % of pre-ischemic values) in WY-treated and untreated hearts exposed to mitochondrial K_{ATP} blocker 5-hydroxydecanoate (5-HD). Values are means ± SEM from 8-10 hearts per group. * P<0.05 vs. C; # P<0.05 relative to 5-HD-untreated hearts. **D.** Levels of conjugated dienes in the myocardium of WY-treated and untreated rats at baseline (BL) and after I/R. Values are means ± SEM from 5-6 hearts per group. * P<0.05 vs. C; # P<0.05 relative to BL.

remote PC induced by renal I/R (Lotz *et al.* 2011), it is still not sufficiently elucidated which downstream mechanisms may underlie PC-like protection conferred by PPAR-α activation by its synthetic ligands.

In our previous study (Ravingerová *et al.* 2012), we found markedly upregulated gene expression of PPAR-α coupled with an increased cardiac Akt phosphorylation after 5-days administration of WY. Matsui and Rosenzweig (2005) suggested that acute activation of PI3K/Akt, both *in vitro* and *in vivo* can promote cardiomyocyte survival and function. Moreover, activation of PI3K/Akt and its downstream targets, such as PKC and eNOS, plays a crucial role in different cardioprotective interventions by mediating antiapoptotic and antioxidative effects (Tong *et al.* 2000, Matejíková *et al.* 2009a, Murphy and Steenbergen 2011).

In the present study, a 2-fold increase in eNOS expression (Fig. 1A) was observed following 5-days treatment with PPAR-α activator that could be related to enhanced Akt phosphorylation (Ravingerová *et al.* 2012) as its downstream mechanism in the hearts of WY-treated rats. A major role of eNOS/NO signaling in cardioprotection has been proven not only in the classical studies of the delayed phase of IPC (Bolli *et al.* 1997), but a positive role of eNOS/NO pathway due to its involvement in the mechanisms of protection triggered by short-term cardiac adaptation by IPC has been also reported (Andelová *et al.* 2005). In addition, it has been shown that eNOS is required for the effect of classical IPC, and its deletion abolishes IS-limiting effect (Talukder *et al.* 2010). Finally, in the recent study (Yang *et al.* 2013), inhibition of PI3K/Akt abolished IPC-induced post-ischemic myocardial recovery and salvage and increase in eNOS expression and phosphorylation. Thus, our finding of increased eNOS expression in the WY-treated myocardium may be relevant to the cardioprotective effects afforded by PPAR-α activation.

The most important finding of the present study is that cardioprotective effect of WY on the lethal injury and postischemic stunning was blunted by administration of mitoK_{ATP} channels blocker 5-HD (Fig. 1B,C) indicating an important role of mitoK_{ATP} channels opening in cardioprotective effects of PPAR-α agonist. It is in agreement with the studies documenting the role of mitoK_{ATP} channels opening in different cardioprotective interventions induced either by classical IPC (Matejíková *et al.* 2009b) or PC induced by K_{ATP}-opener diazoxide (Forbes *et al.* 2001, Matejíková *et al.* 2009a), by post-conditioning (Jin *et al.* 2012), or by delayed effect (24 h after administration) of kappa-opioid receptor agonist (Chen *et al.* 2003) and remote (limb ischemia) PC (Wu *et al.* 2011), as well as by adaptation to chronic intermittent hypoxia (Neckář *et al.* 2002).

In the initial phase of adaptation, various interventions, such as different protocols of hypobaric hypoxia (Kolář *et al.* 2007, Wang *et al.* 2011, Singh *et al.*

2013) or mitoK$_{ATP}$ channels opening (Forbes *et al.* 2001, Matejíková *et al.* 2009b) have been shown to be linked with an increased production of ROS activating pro-survival signaling pathways such as PI3K/Akt (Qin and Chock 2004), PKC-ε, and/or glycogen synthase kinase-3β (Wang *et al.* 2011) leading to functional recovery and infarct size limitation. Interestingly, Thuc *et al.* (2010) reported that treatment with pravastatin up-regulated ROS generation in isolated perfused rat heart prior to ischemia and that infarct size-limiting effect was attenuated by N-acetylcysteine, 5-HD, L-NAME or staurosporine indicating the involvement of ROS in the pro-survival cascades activated by pravastatin. Similarities between the pleiotropic effects of statins and PPAR-α agonists suggest a mechanistic link between these two classes of drugs and similarity in their effects (Paumelle and Staels 2008). This is in line with our findings of increased myocardial levels of CD after 5-days treatment with PPAR-α ligand (Fig. 1D). Moreover, pirinixic acid has been shown to increase ROS production *via* induction of NADPH oxidase, however, in another cell type and different setting (Teissier *et al.* 2004, Sharifpanah *et al.* 2008). Thus, this is a novel finding, since PC-like protective effects of hypolipidemic drugs have not been yet considered in relationship with mitoK$_{ATP}$ channels opening and ROS production in cardiomyocytes.

Protective effect of PPAR-α ligand was accompanied with a reduction in the levels of CD in the post-I/R phase as compared with those at baseline in WY-treated group (Fig. 1D), which was in contrast to increased post-I/R lipid peroxidation in the controls. This finding is not unexpected, since activation of PPAR-α may exhibit, besides metabolic effects, non-metabolic antioxidant effects *via* inhibition of the activity of other transcription factors, such as nuclear factor-kappa B (NF-κB) that plays an important role in oxidative stress evoked by I/R in different organs (Yue *et al.* 2003, Collino *et al.* 2006). Other, PPAR-independent mechanisms may be related to the similarity of effects of pirinixic acid with IPC with respect to triggerring of pro-survival cardioprotective cascade, in particular, activation of PI3K/Akt and its downstream targets, with the final effect in mitochondria and reduction of ROS generation (Murphy and Steenbergen 2011). Moreover, it has been suggested by Vanden Hoek (2000) that protection afforded by PC is associated with attenuation of the oxidant burst at reperfusion, regardless of the method by which PC is triggered.

In conclusion, blocking of mitoK$_{ATP}$ channels with 5-HD abolished protection against lethal myocardial injury and contractile dysfunction in isolated rat hearts conferred by 5-days treatment of rats with PPAR-α synthetic agonist pirinixic acid. The latter also increased cardiac eNOS protein expression and levels of CD, and reduced lipid peroxidation during ischemia in the myocardium of WY-treated rats. Thus, taken together our results indicate that IS-limiting and anti-stunning effects can be achieved by PPAR-α activation and appear to be mediated by mitoK$_{ATP}$ channels opening and by attenuated ROS production during I/R.

Conflict of Interest

There is no conflict of interest.

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