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Anti-ischaemic activity of an antioxidant aldose reductase inhibitor on diabetic and non-diabetic rat hearts

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Abstract

Objectives Many observations report the cardioprotective effects of inhibitors of aldose reductase in different models of ischaemia–reperfusion injury in diabetic myocardium. In this paper, the inhibitory effects of the new pyrido[1,2-a]-pyrimidin-4-one derivative PPO, whose aldose reductase-inhibitory and antioxidant effects were shown in a previous study, were evaluated.

Methods The effect of PPO was evaluated on aldose reductase from hearts of diabetic and non-diabetic rats, and compared with that of the reference drug epalrestat. Moreover, the two drugs were tested on isolated and Langendorff-perfused diabetic and non-diabetic hearts submitted to ischaemia–reperfusion cycle.

Key findings Epalrestat showed equivalent levels of potency in inhibiting the activity of the enzyme in the diabetic and in the non-diabetic hearts. On the contrary, the inhibitory potency of PPO was decreased in the diabetic organs. In the diabetic hearts submitted to ischaemia–reperfusion, an increased level of heart aldose reductase activity was recorded, and both PPO and epalrestat produced cardioprotective effects, suggesting that aldose reductase is deeply involved in the process of ischaemia–reperfusion injury in diabetic myocardium. In non-diabetic hearts, where aldose reductase has a lower activity, epalrestat failed to produce significant protection, while PPO still maintained cardioprotective effects, which may be reasonably attributed to useful ‘ancillary’ effects – such as antioxidant activity – independent from the aldose reductase inhibition.

Conclusions Therefore PPO, a new molecule endowed with both aldose reductase-inhibitory effects and antioxidant activity, may represent the prototype of a new class of multitarget drugs, focused on two different steps deeply involved in the pathogenesis of ischaemic injury of diabetic hearts.

Keywords aldose reductase; antioxidant effect; cardiovascular complications; diabetes; myocardial ischaemia

Introduction

Aldose reductase (AR), belonging to the family of aldo-keto reductase enzymes, catalyses an NADPH-dependent reduction of aldo-sugars and of a number of aldehydes, and represents the first effector of the polyol pathway.[1–3] In this pathway, which can be viewed as an alternative route for glucose metabolism, AR converts glucose to sorbitol, which in turn is converted to fructose by sorbitol dehydrogenase.

In diabetic patients and, more generally, in conditions of hyperglycaemia, AR activity is increased[4] and the polyol pathway is potentiated, leading to accumulation of end products and depletion of important cofactors, such as NADPH and NAD^+. This metabolic impairment seems to play a pivotal role in the development of many complications associated with the diabetic status, such as peripheral neuropathy, damage of lens and retina, nephropathy, etc.

Consistently, given this pathogenetic implication of AR, its inhibition has been proposed as a promising strategy for the treatment of several diabetes-related disorders.[2,5–7]

The impact of diabetes on the cardiovascular system is dramatic, being associated with a plethora of microvascular and macrovascular complications, mainly due to heavy biochemical alterations, such as oxidative stress, impairment of glycaemic and lipaemic

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parameters and vascular inflammation, finally converging against the vascular endothelium and producing a deleterious endothelial dysfunction.[8,9] As a consequence, cardiovascular and cerebrovascular accidents, such as myocardial ischaemia and stroke, represent the main causes of death in diabetic people.

The role of AR in these diabetes-related cardiovascular disorders is quite controversial. On one hand, an increased activity of AR, with subsequent detoxification of reactive aldehydes such as 4-hydroxynonenal deriving from ischaemic oxidative stress, has been viewed as a factor potentially contributing to the late phase of ischaemic pre-conditioning, an endogenous and complex self-defence mechanism ensuring an increased resistance of myocardium against ischaemia.[10–13] On the other hand, in conditions associated with hyperactivation of AR and acceleration of the polyol pathway (such as the diabetic status), AR has been deemed a key factor for the development of vascular damage.[2,14–16] Recent studies on transgenic mice over-expressing AR furnished clear and convincing evidence that AR is an important component of myocardial ischaemia–reperfusion injury.[17] Moreover, these studies demonstrated that AR over-expression is related to an increased opening of the mitochondrial permeability transition pore,[17] which is a key step in ischaemia–reperfusion cell death.[18]

In agreement with this latter hypothesis relating to a negative role of AR in myocardial ischaemia–reperfusion injury, many observations reported a clear cardioprotective effect of pharmacological inhibition of AR in different experimental models of myocardial ischaemia–reperfusion,[6,17,19,20] indicating a pragmatic strategy for pharmacological management of heart ischaemia, which is one of the most dangerous risks associated with diabetes. In particular, convincing results clearly demonstrated that diabetic rat hearts show an impaired glucose metabolism closely related to an elevated polyol pathway; more importantly, after ischaemia–reperfusion cycles, AR inhibition leads to better preservation of ATP and NAD^+ and reduced sorbitol formation. Further confirmation of the detrimental role of increased AR activity in ischaemic hearts has been elegantly produced by Hwang and colleagues[21] and by Iwata and colleagues.[7] These authors reported that ischaemia–reperfusion injury is higher in hearts from transgenic mice over-expressing AR than in those of wild-type mice. Again, the pharmacological inhibition of AR determined a strong cardioprotective effect on hearts over-expressing AR, with improved glucose metabolism, preservation of ATP levels and an overall reduction of functional and biochemical markers of ischaemia–reperfusion damage.

Hyperglycaemic status is also associated with a dramatic increase of reactive oxygen species (ROS) and the consequent oxidative stress is another key factor for the development of cardiovascular diabetic complications. Also, an increased level of ROS during myocardial ischaemia–reperfusion is one of the most deleterious effectors of cell injury. Consistently, many diabetic complications (in particular the cardiovascular ones) can be advantageously attenuated by treatment with antioxidant agents, such as vitamins C and E, trolox, etc.[22,23]

In a previous work, a series of new pyrido[1,2-a]-pyrimidin-4-one derivatives was described. These compounds were designed to exhibit AR-inhibiting properties, with no (or poor) effect on other enzymes of the aldo-keto reductase family.[24] Moreover, the presence of relevant structural functionalities, such as phenolic or catecholic hydroxyl groups, conferred to some compounds of that series additional antioxidant properties. In particular, 2-(3,4-dihydroxyphenyl)-6-hydroxypyrido[1,2-a]pyrimidin-4-one (PPO; Figure 1) showed submicromolar orders of potency (Ki ~ 0.1 μM) in inhibiting AR extracted from rat lens, negligible effects on aldehyde reductase obtained from rat kidney (Ki > 10 μM) and strong antioxidant activity, thus representing the most interesting compound of the series. Since these biological effects of PPO seem to fit an optimal pharmacodynamic profile for diabetic heart complications, this work examined the pharmacological activity of PPO at the cardiac level. In particular, the experimental work was focused on the evaluation of the possible inhibitory effects of PPO on cardiac AR and of its potential cardioprotective properties on diabetic rat isolated hearts submitted to ischaemia–reperfusion.

Materials and Methods

Animals

Male Wistar rats, 250–350 g (Harlan, Milan, Italy), were housed and cared for in conformity with the Guidelines of the European Community Council Directive 86/609, adopted by Italian law D.L. 116/92. The procedures were approved by the ethical committee of the University of Pisa.

Diabetes induction

When required for experimental procedures, diabetes was induced with a single intraperitoneal injection of

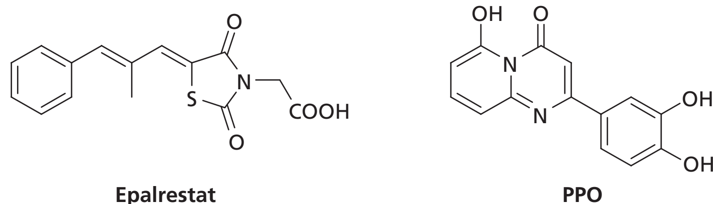

Figure 1 Chemical structures of the aldose reductase inhibitors epalrestat and PPO

streptozotocin (STZ) 50 mg/kg dissolved in citrate buffer (vehicle, pH 4.6). Blood samples for measurement of glycaemia were taken from the tail vein 24 h after the STZ injection and values of fasting glycaemia > 200 mg/dl were considered as indicative of successful induction of diabetes, while STZ-treated rats showing fasting glycaemia levels < 200 mg/dl were excluded from the study. Fourteen days after the diabetes induction, diabetic rats showed fasting glycaemia levels of 361 ± 24 mg/dl, while the vehicle-treated non-diabetic rats exhibited glycaemic levels of 71 ± 6 mg/dl. The experimental protocols were carried out 14 days after the treatment with STZ or vehicle.

Inhibitory effect on cardiac aldose reductase

Inhibition curves for epalrestat and PPO were obtained on hearts of diabetic and non-diabetic rats, not submitted to ischaemia–reperfusion. Hearts were excised from rats anaesthetised with sodium pentobarbital (100 mg/kg, i.p.) and perfused for 15 min on a Langendorff apparatus with Krebs solution (composition in mM: 118 NaCl, 4.8 KCl, 1.2 K_2HPO_4, 1.6 $CaCl_2$, 1.2 $MgSO_4$, 25 $NaHCO_3$ and 11.5 glucose) at 37°C saturated with 95% O_2–5% CO_2, to clean up the coronary blood vessels. Then, the left ventricular tissue was ice-cool homogenised with bi-distilled water (1 : 4 w/v) and centrifuged for 1 h at 10 000*g* at 4°C. The supernatant fraction was diluted 1 : 20 (v/v) in phosphate buffer (pH 6.2) and used for enzyme assay.

The AR activity was measured spectrophotometrically by estimating NADPH oxidation, based on the decrease in absorbance at 340 nm for 5 min at 25°C. The reaction mixture contained in phosphate buffer (pH 6.2): sample, glyceraldehyde 0.1 mM, the AR-inhibitor 5-[(1E,2E)-2-methyl-3-phenylpropenylidene]-4-oxo-2-thioxo-3-thiazolidineacetic acid (epalrestat, 10^{-10} to 10^{-5} M), the test compound PPO (10^{-9} to 10^{-4} M) or vehicle (dimethyl sulfoxide, DMSO); the assay was started by addition, after 10 min incubation, of the enzymatic cofactor NADPH 0.1 mM.

Glyceraldehyde replaced by bidistilled water was used as a blank.

Protein concentration in the supernatant fraction of cardiac tissue was measured by Bradford's method, using bovine serum albumin as a standard for calibration curves.

Increasing concentrations of AR inhibitors allowed preparation of an inhibition curve, which was analysed by computerised methods (software GraphPad Prism 4.0) to calculate the IC50 value (molar concentration of the test compound evoking half inhibition of the AR activity), which was converted into the dissociation constant (expressed as mean ± SEM from $n > 6$ experiments) through the Cheng–Prusoff algorithm.

The inhibition curves were statistically analysed by two-way analysis of variance. Each curve represented the mean from $n > 6$ experiments. $P < 0.05$ was considered as an indicator of significant difference.

Isolated heart preparation

Two hours before the experimental procedure, rats received an intraperitoneal injection of the AR inhibitors epalrestat (40 mg/kg) or PPO (40 mg/kg) or of vehicle (DMSO).

Rats were heparinised (100 IU, i.p.) to prevent blood clotting and anaesthetised with sodium pentobarbital (100 mg/kg, i.p.).

After the opening of the chest, the heart was quickly excised and placed in 4°C Krebs solution (composition in mM: 118 NaCl, 4.8 KCl, 1.2 K_2HPO_4, 1.6 $CaCl_2$, 1.2 $MgSO_4$, 25 $NaHCO_3$ and 11.5 glucose) equilibrated with 95% O_2–5% CO_2 to stop the contraction and to reduce oxygen consumption. Then, hearts were mounted within 2 min in a Langendorff apparatus (Radnoti, Monrovia, USA) and perfused at 37°C at constant pressure (70–80 mmHg) with Krebs solution bubbled with 95% O_2–5% CO_2.

Left ventricular developed pressure (LVDP) and heart rate (HR) were measured using a water-filled latex balloon, placed in the left ventricle via the mitral valve, connected to a pressure transducer and then to a data acquisition system (Biopac Inc., Goleta, USA), to record continuously the functional parameters. The volume was adjusted to achieve a stable left ventricular end-diastolic pressure of 5–10 mmHg during initial equilibration. The functional parameter RPP (product pressure rate) was calculated as LVDP × HR. After 30 min of equilibrating time (pre-ischaemic time), hearts were subjected to 30 min global ischaemia, followed by 120 min reperfusion.

The values of post-ischaemic RPP, recorded in the reperfusion time, were calculated as a percentage (RPP %) of the pre-ischaemic values, expressed as mean ± SEM. The curves of RPP% vs time were statistically analysed by two-way analysis of variance. Each curve represents the mean from $n > 12$ experiments. $P < 0.05$ was considered as an indicator of significant difference.

Planimetric determination of ischaemia–reperfusion injured myocardium

Left ventricles of hearts from some of the ischaemia–reperfusion experiments were dried, frozen and cut into two or three transverse slices from the apex to base of equal thickness (about 2 mm). The slices were then incubated in a 1% 2,3,5-triphenyltetrazolium chloride (TTC) solution in a phosphate buffer (pH 7.4) at 37°C for 20 min. After incubation overnight in 10% formaldehyde, the slices were photographed and the infarct area (Ai) was calculated by planimetric analysis using an image analyser program (The GIMP 2). The size of the injured area was expressed as a percentage of the whole left ventricle area (Ai/Atot %), expressed as mean ± SEM from $n > 6$ experiments. The data were statistically analysed by one-way analysis of variance. $P < 0.05$ was considered as an indicator of significant difference.

Activity of cardiac aldose reductase

Left ventricles of hearts from some of the ischaemia–reperfusion experiments were used to test the AR activity, to confirm the effective inhibition of the enzyme by epalrestat and PPO, which were administered *in vivo* 2 h before the experiments. The extraction procedure and the assay of the enzyme were performed as reported above. AR activity values were calculated as a percentage of the activity recorded in the hearts of vehicle-treated non-diabetic rats and

were reported as the mean ± SEM from $n > 6$ experiments. The data were statistically analysed by one-way analysis of variance. $P < 0.05$ was considered as an indicator of significant difference.

Results

Inhibition of aldose reductase from diabetic and non-diabetic rat hearts

Pre-incubation with increasing concentrations of PPO or the reference drug epalrestat produced a concentration-dependent progressive inhibition of activity of the enzyme extracted from hearts of diabetic and non-diabetic rats (Figure 2). Of particular note, in heart homogenates from vehicle-treated (non-diabetic) rats, epalrestat exhibited a pKi value of 7.19 ± 0.10 ($n = 8$) while PPO showed a slightly lower level of potency, with a pKi of 6.33 ± 0.13 ($n = 10$), which is in good agreement with the sub-micromolar level of potency previously observed in experiments performed on AR from rat lens.[24] In heart homogenates from STZ-treated (diabetic) rats, the inhibitory properties of epalrestat were almost equivalent to those exhibited in non-diabetic conditions (pKi = 7.50 ± 0.25) ($n = 6$), while PPO showed a significant decrease of inhibitory potency (pKi = 5.11 ± 0.12) ($n = 8$).

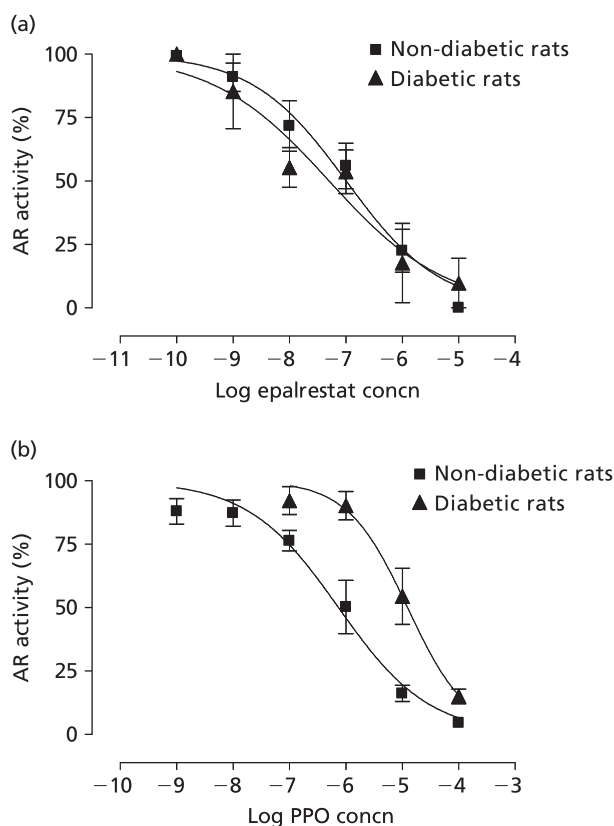

Figure 2 Inhibition of aldose reductase. Aldose reductase (AR) inhibition curves for epalrestat (a) and PPO (b) in homogenates obtained from hearts of non-diabetic or diabetic rats. Data are means ± SEM, $n > 6$.

Protective effect in diabetic and non-diabetic rat hearts submitted to ischaemia–reperfusion

Global ischaemia in hearts from non-diabetic rats caused a dramatic reduction of inotropic function in the reperfusion period, reflected by low levels of RPP % (Figure 3), and a wide extension of injured tissue (Ai/Atot = 38 ± 4). Pre-administration of epalrestat to non-diabetic rats did not produce any significant recovery of functional parameters (Figure 3); the planimetric parameters of ischaemic injury (Ai/Atot = 28 ± 4) were not significantly lower than those recorded in the vehicle-treated rats. On the contrary, PPO produced evident cardioprotective effects in this experimental model of ischaemia–reperfusion in non-diabetic rat hearts, with a high increase in the post-ischaemic functional parameters (Figure 3) and a significant reduction of the damaged areas (Ai/Atot = 21 ± 6).

Global ischaemia in hearts from diabetic rats pre-treated with vehicle caused a clear damage, almost equivalent to that observed in non-diabetic hearts, well represented by a dramatic loss of inotropic function in the reperfusion period and by a wide extension of injured tissue (Ai/Atot = 39 ± 4)

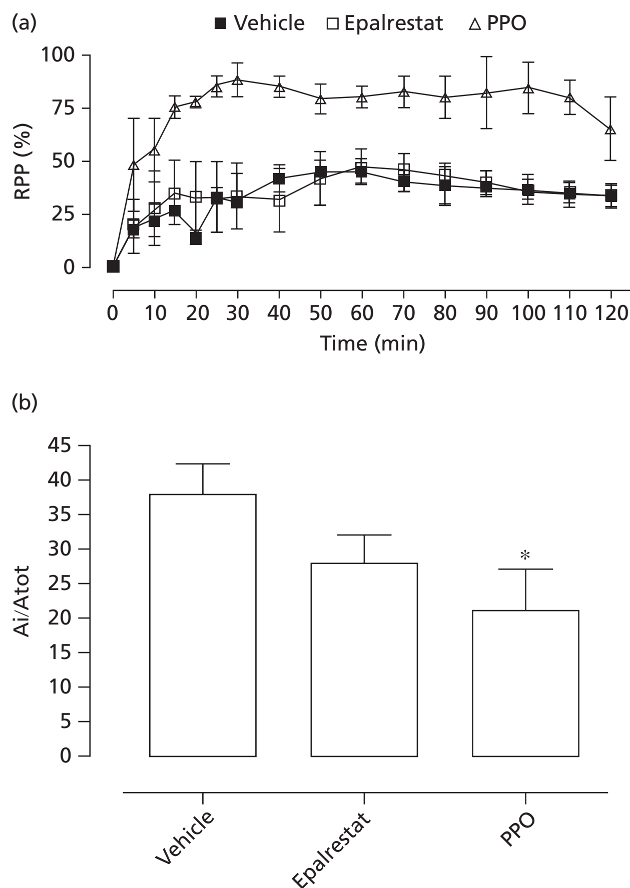

Figure 3 Protective effects in non-diabetic hearts submitted to ischaemia–reperfusion. (a) Post-ischaemic functional parameters of RPP % recorded, during the reperfusion time, in hearts of non-diabetic rats pre-treated with vehicle, epalrestat or PPO. (b) Size of injured areas (expressed as Ai/Atot), observed in the left ventricles of non-diabetic rats pre-treated with vehicle, epalrestat or PPO. Data are means ± SEM, $n > 12$. *$P < 0.05$ compared with vehicle.

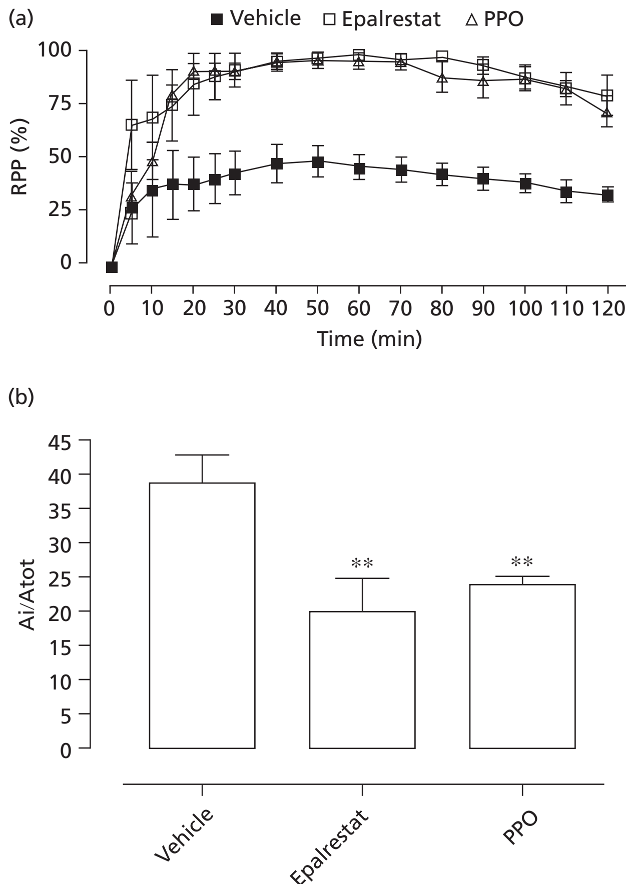

Figure 4 Protective effects in diabetic hearts submitted to ischaemia–reperfusion. (a) Post-ischaemic functional parameters of RPP % recorded, during the reperfusion time, in hearts of diabetic rats, pre-treated with vehicle, epalrestat or PPO. (b) Size of injured areas (expressed as Ai/Atot), observed in the left ventricles of diabetic rats pre-treated with vehicle, epalrestat or PPO. Data are means ± SEM, $n > 12$. $^{**}P < 0.01$ compared with vehicle.

(Figure 4). The hearts of diabetic rats pre-treated with epalrestat attained satisfactory recovery of the post-ischaemic inotropic function and showed a significant reduction of injured tissue (Ai/Atot = 20 ± 5) (Figure 4).

These effects of epalrestat were completely reproduced by PPO, which allowed diabetic rat hearts to achieve a good recovery of the inotropic function, and a significant reduction of injured tissue (Ai/Atot = 24 ± 1) (Figure 4).

Activity of aldose reductase in diabetic and non-diabetic rat hearts submitted to ischaemia–reperfusion

The evaluation of cardiac AR activity at the end of the ischaemia–reperfusion cycle, calculated as a percentage of the value recorded in vehicle-treated non-diabetic rats, allowed the observation that the value recorded in diabetic hearts of vehicle-treated rats was 256 ± 11% (Figure 5). The administration of the inhibitors caused a significant reduction of the activity of AR in non-diabetic and diabetic hearts submitted to ischaemia–reperfusion. In particular, the activity recorded in

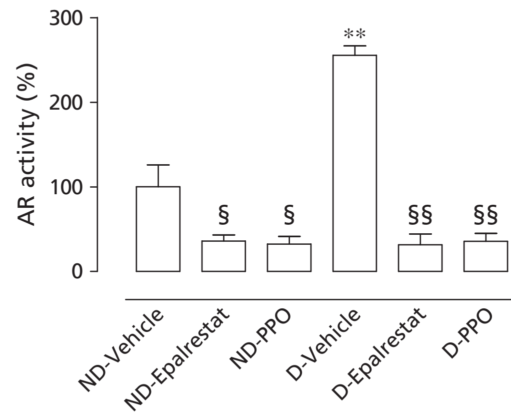

Figure 5 Aldose reductase activity in hearts submitted to ischaemia–reperfusion. Values of aldose reductase (AR) activity recorded in vehicle-treated diabetic (D-Vehicle) and non-diabetic (ND-Vehicle) rat heart submitted to ischaemia–reperfusion, or in drug-treated diabetic and non-diabetic rat hearts submitted to ischaemia–reperfusion. The level of activity is expressed as a % of the mean value recorded in ND-Vehicle rats. Data are means ± SEM, $n > 6$. $^{**}P < 0.01$ compared with ND-vehicle; $^{§}P < 0.05$, $^{§§}P < 0.01$ compared with the corresponding vehicle-treated sample.

epalrestat-treated non-diabetic and diabetic rats was 36 ± 7 and 32 ± 13, respectively, while the activity recorded in PPO-treated non-diabetic and diabetic rats was 32 ± 9 and 36 ± 9, respectively (Figure 5).

Discussion

The potential inhibitory effects of AR by the pyrido[1,2-a]-pyrimidin-4-one derivative PPO have been investigated in diabetic and non-diabetic rat hearts, and compared with those of the well-known drug epalrestat. As previously observed in AR obtained from rat lens,[24] PPO inhibited cardiac AR of non-diabetic rats at a potency about 10-fold lower than that of epalrestat. The diabetic status did not influence the sensitivity of the enzyme for the inhibition by epalrestat, while the potency of PPO in inhibiting AR from diabetic rats was subjected to a significant reduction. As concerns the cardioprotective effects deriving from AR inhibition, epalrestat conferred to diabetic hearts an increased resistance against the ischaemic injury, leading to a good recovery of cardiac inotropism and strongly reducing the extension of ventricular necrotic damage. On the contrary, epalrestat could not attenuate significantly the myocardial injury due to ischaemia–reperfusion in non-diabetic hearts. Such a conflicting behaviour of epalrestat can be explained by the data concerning AR activity, which were measured in hearts deriving from the experimental protocols of ischaemia–reperfusion. In agreement with the findings of other research teams, reporting that diabetic status leads to a hyperactivation of AR,[4] our study of hearts from diabetic rats showed a potentiated activity of this first step of the polyol pathway, with an AR relative activity more than 2-fold higher (256 ± 11%) than that observed in non-diabetic controls. Both in diabetic and in non-diabetic hearts, pre-treatment with epalrestat produced a marked inhibition of the enzyme activity, thus indicating that the lack of cardioprotective

activity of epalrestat in the non-diabetic heart is not due to a pharmacological ineffectiveness on its target, but rather suggesting that this enzyme is not significantly involved in the pathogenesis of the ischaemia–reperfusion damage of non-diabetic myocardium, probably because of its relatively low activity in such conditions.

Despite PPO showing a lower inhibitory potency in the enzymatic assay, the pre-administration of PPO (like epalrestat) caused a significant reduction of AR activity recorded in both diabetic and non-diabetic hearts submitted to ischaemia–reperfusion. Consequently, it was not surprising that its cardioprotective activity against the ischaemic injury of diabetic rat hearts was almost equivalent to that of epalrestat. On the contrary, an interesting result emerged from the evaluation of PPO in non-diabetic hearts submitted to the ischaemia–reperfusion cycle, where PPO was able to induce clear cardioprotective effects while epalrestat was almost ineffective. Of course, an exhaustive explanation of such a peculiar feature of PPO cannot be given, although it can be hypothesised that this can reside in possible 'ancillary' effects of the molecule. As reported above, the antioxidant properties of PPO have been clearly reported in a previous study on rat brain homogenates. Given the important role played by oxidative damage in the progress of the injury due to ischaemia–reperfusion, it can be hypothesised that this antioxidant 'ancillary' function can be viewed as an additional anti-ischaemic mechanism, useful to potentiate the whole cardioprotective profile of PPO. Such a speculative hypothesis will be evaluated in future experimental approaches, in which we will evaluate the influence of PPO on the oxidative injury caused by ischaemia–reperfusion in different and integrated experimental models, useful for a more detailed characterisation of anti-ischaemic drug effects.[25]

Conclusions

In conclusion, as above discussed, a 'pure' aldose reductase inhibitor can be viewed as a 'Janus', with its two antithetic faces: the beneficial reduction of the polyol pathway and detrimental abolition of the detoxification role of AR. Actually, a possible correlation between enzyme inhibition and vascular oxidative stress has already been suggested; also, a possible prevention of late-phase ischaemic preconditioning has been linked to pharmacological treatment with AR inhibitors.[26] Therefore, the development of innovative agents, able to inhibit AR-mediated deleterious pathways without affecting AR-mediated detoxification, are considered to be useful agents for treatment of micro- and macrovascular diabetes-associated complications.[26] Unfortunately, both of these aspects are intrinsic to AR activity, and hence they cannot be separated by any 'pharmacological selectivity'. However, another pharmacological strategy can be advantageously developed by conferring to an AR inhibitor additional pharmacodynamic features, able to correct or attenuate the potentially negative aspects intrinsically bound to AR-inhibition. For these purposes, the project of AR inhibitors endowed also with antioxidant properties has been an important field of research in medicinal chemistry, which led to the synthesis of PPO, as well as of other antioxidant AR inhibitors exhibiting a pyridoindole moiety.[27] These compounds seem to possess a profile particularly useful for 'neutralizing' the potential detrimental side-effect of AR-inhibition.

This preliminary study suggested that PPO can be viewed as an interesting chemotype of new molecules, endowed with both AR-inhibitory effects and antioxidant activity; future experimental work will aim to evaluate this molecule, as a lead compound of a rational multi-target strategy, focused on two different steps deeply involved in the pathogenesis of ischaemic injury in diabetic hearts.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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