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The Antianginal Agent Ranolazine Inhibits Mitochondrial β-Oxidation Pathway

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Abstract

Backgrounds: Ranolazine had two main functions: blocking cardiac late sodium channels and / or inhibition of 3-ketoacyl coenzyme A thiolase, an important enzyme in ß-oxidation of fatty acids. Diabetic cardiomyopathy is a complication, defined as cardiac dysfunction without the involvement of epicardial vessels. In this study, the effect of ranolazine on fatty acid oxidation was investigated in diabetic rats induced by streptozotocin.

Methods: After 8 weeks of diabetic induction, the effect of ranolazine on fatty acid oxidation rate was studied. Statistical data were analyzed using Mann-Whitney test.

Results: The activity of ß-oxidation enzymes were inhibited by ranolazine in the normal rat hearts significantly, while no significant inhibition was seen in diabetic ones.

Conclusion: Our data suggest that the clinical efficacy of ranolazine in diabetic patients is associated with a mechanism other than inhibition of the ß-oxidation pathway activity, although various hypotheses exist in the literature.

Keywords: Ranolazine; Cardiac fatty acid oxidation; Diabetes

Introduction

The incidence of mortality from cardiovascular diseases is high in diabetic patients. Diabetic cardiomyopathy is a complication, manifested as diastolic dysfunction in earlier- and systolic dysfunction in later stages [1]. In diabetic cardiomyopathy, metabolism of substrate shifts from glucose to the higher level of free fatty acids due to hyperlipidemia and insulin resistance. Diabetes is marked by decreases in the intensity of glucose transport, glucose oxidation and phosphorylation, plus decreases in ATP levels in cardiac myocytes [2]. Fatty acid oxidation produces 90% of ATP in diabetic hearts. Increase of free fatty acids, by the activation of PPAR- α receptors, increase the activity and expression of pyruvate dehydrogenase kinase 4 and stimulates mitochondria to reuptake fatty acids, and inhibits pyruvate dehydrogenase complex [3]. As the rate of glycolysis decreases, production of lactate and H^+ increases that leads to intracellular acidosis. Under this condition, reduced activity of Na^+ / K^+ATPase and Na^+- Ca^{2+} exchanger result sodium and calcium overload and may enhance myocyte damage [4]. It is important to find some new treatments that partially inhibit extensive fatty acid ß-oxidation and promote glucose consumption in diabetic heart.

Ranolazine, an antianginal drug, acts through blocking cardiac late sodium channels and/or inhibition of 3-ketoacyl coenzyme A thiolase, an important enzyme in β-oxidation of fatty acids [5]. In heart failure, late sodium current increases and membrane sodium-calcium exchanger shifts to reverse mode that leads to intracellular calcium increase. Ranolazine treatment can inhibit the late sodium channels in myocardial dysfunction [6]. One study has been reported that chronic heart failure (using micro emboli in dog) with IV injection of ranolazine increased ventricular contractility and ejection fraction [7]. Ranolazine has improved contractility in stressed conditions such as low flow ischemia, ischemia-reperfusion and heart failure, but some of the early studies demonstrated that ranolazine had no effect on cardiac function in the normoxic heart [7].The effects of ranolazine on the activities of β- oxidation enzymes in diabetic cardiomyopathy have not yet been studied. In diabetes, metabolism tends to use fatty acid for energy production, which produces cardiac dysfunction thus; ranolazine may be effective in this situation. Therefore, we studied the effect of ranolazine at concentrations 3 µM - 100 µM on fatty acid β-oxidation enzymes in diabetic and normal rat hearts.

Materials and Methods

Chemicals

STZ (streptozotocin), NAD^+, Coenzyme A and Palmitoyl CoA were purchased from Sigma Chemical Company (St. Louis, USA) and Acyl CoA dehydrogenase was obtained from WaKO (Japan). Ranolazine was obtained from LKT Laboratories (USA) and other chemicals were purchased from Merck Chemical Company (Germany).

Experimental protocol

14 adult male Sprague–Dawley rats weighing about 200 g - 250 g were obtained from Animal Care Center of Shiraz University of Medical Sciences. The animals were kept in the laboratory conditions of 24°C ± 2°C with a 12 h light / dark alternating cycle with free access to water and standard diet. The animal care protocols were done according to the animal ethical rules of the Council for Shiraz University of Medical Science. The rats were randomly divided into 2 groups of the control and diabetic groups containing 7 rats each one. The rats were adjusted for age and sex in this study to prevent these confounding factors.

Induction of diabetes

Diabetes was induced in overnight-fasting rats of diabetic group by a single intraperitoneal injection of STZ (60 mg/kg) dissolved in the citrate buffer (0.1 M, pH 4.5) as the vehicle, whereas the rats in the control group received only the vehicle. Fasting blood glucose levels were measured 7- days after STZ injection by an Accu-Check Active glucometer (Roche, Germany) in the blood were drawn from the tail vein. Diabetes was confirmed in the rats if blood glucose > 350 mg/dl. The rats in 2 groups kept for 8 weeks in the laboratory conditions as mentioned before.

Mitochondrial membrane preparation

The two subgroups (control and diabetic) were euthanized with pentobarbital (100 mg/kg body weight) and underwent thoracotomy. The hearts were immediately removed, weighted and sliced to small parts. Then the particles of all rat's hearts were rinsed and homogenized with the buffer contained: 5 mM Tris - HCl buffer (pH = 7.4), 0.25 M sucrose, 1 mM EDTA at 4°C. Homogenates were then centrifuged at 800 g for 10 min to remove nuclei and cell debris. The supernatant was further centrifuged at 6000 g for 15 minutes and resulting mitochondrial pellets were then dissolved in Tris buffer and frozen in liquid nitrogen. The frozen pellets were thawed and sonicated for 15 seconds, and centrifuged at 100,000g for 1 h. Finally the pellet containing mitochondrial membrane was separated, dissolved in 1 ml Tris buffer and refrigerated at -70°C until further analysis [8].

Enzymes assays

Mitochondrial membrane fraction contains tri-functional protein (TFP) that functions as an important enzyme in ß-oxidation of free fatty acids as shown in Figure 1.

Initially acyl CoA dehydrogenase produces the substrate for entry into the TFP complex. TFP consists of a multimeric α- and β-subunit complex; α-subunit contains the enoyl CoA hydratase and L-3-hydroxyacyl CoA dehydrogenase and β-subunit contains 3-ketoacyl CoA thiolase activity [9]. TFP activity was determined by spectrophotometric methods. Trans-Δ^2-enoyl CoA as the substrate of enoyl CoA hydratase of TFP was formed in the reaction mixture contained 0.05 mM palmitoyl CoA in Tris- HCl 100 mM (pH = 7.6) by commercial acyl CoA dehydrogenase (6.6 unit/ml). Increase of absorbance at 263 nm indicates the activity of enzyme. Activity of enoyl CoA hydratase of TFP was measured using the mitochondrial membrane fraction as the source of enzyme and trans-Δ^2-enoyl CoA in the Tris- HCl buffer (100 mM, pH = 9) contained $MgCl_2$ 25 mM, KCl 50 mM and BSA = 0.24 mg/ml at 37°C. Increase of absorbance at 263 nm was monitored as a measure of hydratase activity. L-3-hydroxy acyl CoA dehydrogenate activity was calculated by addition of NAD^+ (200 μM) to the reaction mixture and the increase of absorbance at 303 nm recorded. For 3-ketoacyl CoA thiolase assay, after stability of previous step, 0.13 mM coenzyme A (CoA) was added and decrease of absorbance at 303 nm was considered as the thiolase activity. All the enzyme assays were performed in absence and presence of different concentration of ranolazine (3, 10, 30 and 100 μM). Δ absorbance/min was considered as the individual enzymes activities in each step. Different concentrations of acetyl CoA (100, 500, 1000 μM) were also used as the inhibitior of thiolase. These concentrations are chosen from previously published studies [9]. All experiments were repeated three times and data were normalized for total protein (mg/ml). Protein concentration in the heart mitochondrial membrane fraction was measured by the Bradford method using the bovine serum albumin as a standard.

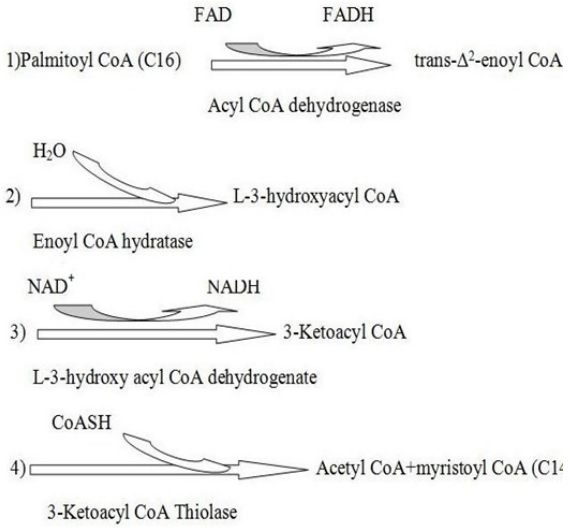

Figure 1: Free fatty acidsβ-oxidation pathway.

Statistical analysis

Results are presented as mean ± SEM of 3 separate experiments. Statistical difference between control and diabetic groups was determined by Mann-Whitney U test. $P < 0.05$ was considered significant. Statistical analysis was performed using SPSS Vers.11 (USA) and Sigma plot Vers.3.1 (USA).

Results

Blood glucose levels and body weights of experimental rats

Fasting blood glucose levels and weight were measured at 56th day in experimental rats. As expected, the diabetic rats showed a significant increase ($P < 0.001$) in blood glucose levels compared to control rats (Table 1). As shown the body weight in diabetic rats after 8 weeks decreased significantly when compared to control rats ($p < 0.01$). Also, heart/body weight ratio in diabetic rats increased significantly at $p < 0.05$ Compared to normal ones (Table 1).

Heart/body weight	FBS (mg/dl)	Heart weight(g)	Body weight(g)	Group
3.6 ± 0.2×10-3	120.4 ± 5.4	1.11 ± 0.04	304.0 ± 9.8	Control
5.4 ± 0.5×10-3	517.8 ± 9.5	1.19 ± 0.07	220.0 ± 10.9	Diabetic
P < 0.05	P < 0.001	NS	P < 0.01	Statistical significance

Table 1: Body weight, heart weight and fasting blood glucose in two groups were measured after 8 weeks. Data showed as Mean ± SE of mean.

There was a significant difference ($p < 0.05$ with Mann-Whitney U test) in protein concentration of mitochondrial membrane fraction of diabetic heart homogenates comparing to control ones (2.58 mg/ml ± 0.16 mg/ml 2.99 mg/ml ± 0.05 mg/ml).

Effects of Ranolazine on Fatty Acid ß-oxidation Enzymes

Mitochondrial membrane fraction was used as the source of ß-oxidation enzymes. Also, ΔOD/mg protein/min reported as an indicator of enzyme activity. Figure 2, demonstrates the effect of ranolazine on enzymes activity of trifunctional protein in control and diabetic rats. There was no significant difference in baseline activities of hydratase, dehydrogenase and thiolase between normal and diabetic rats. In diabetic heart, ranolazine had less inhibitory effect on hydratase compared to normal hearts; in higher concentrations of ranolazine (30 μM and 100 μM), there was statistically significant difference ($p < 0.05$; Figure 2a).

Figure 2b, demonstrates the effect of ranolazine on dehydrogenase activity. In diabetic heart, ranolazine had more inhibitory effect compared to normal hearts but there was no significant difference.

Figure 2c, demonstrates the effect of ranolazine and acetyl CoA on thiolase activity. Inhibitory effect of acetyl CoA was concentration dependant. Also, the assay systems used were CoA- and 3-ketoacyl CoA–dependent and it should be optimized for their concentration as we did. In diabetic heart, ranolazine had less inhibitory effect on thiolase compared to normal hearts, where significant difference ($p < 0.05$) was observed even in therapeutic concentration (10 μM). Acetyl CoA, as an unspecific inhibitor of enzyme, inhibited normal heart more significantly than normal enzymes in all concentrations ($p < 0.05$).

Discussion

The cause of diabetic cardiomyopathy is multi-factorial and it is postulated that myocardial cell dysfunction could presumably result as a consequence of metabolic events. Insulin deficiency makes major abnormalities in glucose and fatty acids metabolism in myocardial cells which activates the ß-oxidation of free fatty acids. These changes could cause disturbances in calcium homeostasis leads to cardiac dysfunction [10]. Thus, ranolazine which inhibits fatty acid ß-oxidation can potentially reverse the uncontrolled derangements in the diabetic heart.

Hyperglycemia induced cardiomyopathy and increased significantly heart to body weight ratio after two months of diabetes induction in experimental rats [11,12] as we shown in the present study. However, the mechanism of this damage on the structure and function of the heart is unclear but stiffness of diabetic hearts was illustrated through substituting cardiomyocytes by interstitial tissues [13,14]. Khazraei et al. also showed no significant difference on diabetic or normal heart microcirculation by laser Doppler technique [15].

As was indicated in Figure 2, the diabetic hearts had the higher levels of the dehydrogenase and thiolase activities than normal hearts at a non-significant level. Ranolazine inhibited all of ß-oxidation enzymes such as thiolase, hydratase and dehydrogenase even in therapeutic concentrations (3-10 μM). Ranolazine had less inhibitory effects on hydratase and 3-keto thiolase activity in diabetic hearts compared to normal hearts. The inhibitory effects of ranolazine on the activity of ß-oxidation enzymes in diabetic rat heart have yet not been reported.

Lopaschuk et al. [8] and MacInnes et al. [9] demonstrated the inhibitory effects of 100 μM ranolazine on thiolase activity in normal rat hearts. We demonstrated 27% inhibition of 3-keto thiolase activity by ranolazine in this concentration.

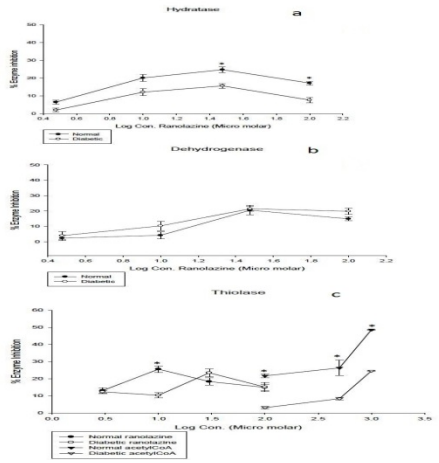

Figure 2: Effect of ranolazine on Hydratase enzyme (a), Dehydrogenase (b),Thiolase (c). Acetyl CoA used as positive control to inhibit thiolase. *P < 0.05 compared to normal hearts. Normal hearts with ranolazine: () and diabetic hearts with ranolazine: (○) and normal hearts with acetyl CoA:()and diabetic hearts with acetyl CoA:(Δ). (N=5).

Our findings was consistent with dose-dependently inhibition by acetyl CoA being as a competitive inhibitor of 3-keto acyl thiolase. Acetyl CoA acted as a sensor of the ß-oxidation pathway that will inhibit the continued flux through this system when products are accumulated.

Mitochondrial dysfunction and less oxidative phosphorylation decreased myocardium energy capacity and caused contractility failure [16]. In one study has been shown that hyperglycemia increased the expression levels of fatty acid ß-oxidation enzymes with consequent up-regulation of mitochondrial electron transport enzymes in rat hearts. In this study, high glucose increased the rate of cell apoptosis and increased the duration of the action potential and elevated level of intracellular cytoplasmic calcium [17].

In another study, ranolazine relaxed the isolated aortic contractions in both normal and diabetic rats by blockade of α-adrenergic receptors and voltage-operated calcium channels [18].Changes in diabetic heart contractility in high concentration may be related to different effects on channels activities [19]. Ranolazine is also different from traditional antianginal drugs and its efficacy in angina is not associated with any hemodynamic effects. Beneficial effects of this drug have been reported in diabetic patients [20] but it seems these effects are not related to its inhibitory effects on ß-oxidation enzymes activity. For future studies, it is recommended that the chronic effects of ranolazine in diabetic animals are investigated in terms of mechanical and electrophysiological heart characteristics and effects on intracellular calcium homeostasis through binding of ranolazine to the mitochondrial permeability transition pore. Further studies will be required to establish molecular targets for the significant effects of ranolazine in diabetic cardiomyopathy.

Conclusion

We used trifunctional protein complex in the crude mitochondrial homogenate of isolated and purified heart tissues in both diabetic and normal rats to assess the ability of ranolazine to inhibit ß-oxidation enzymes activity. We demonstrated that although ranolazine may assist in the recovery of cardiac function after ischemia and angina but diabetic cardiomyopathy could not treated via inhibition of ß-oxidation enzymes. Ranolazine inhibits normal heart enzymes more than diabetic ones in therapeutic concentration.

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