Pharmacological Research 63 (2011) 225–232

Contents lists available at ScienceDirect

Pharmacological Research

journal homepage: www.elsevier.com/locate/yphrs

The effect of losartan treatment on the response of diabetic cardiomyocytes to ATP depletion

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ARTICLE INFO

Article history:
Received 20 June 2010
Received in revised form 22 October 2010
Accepted 4 November 2010

Keywords:
Diabetes
Metabolic intoxication
K_{ATP} channels
Contractile failure
Rigor-type contracture
Ischemia

ABSTRACT

The present work aimed to investigate the effect of losartan treatment of healthy and diabetic rats on cardiomyocyte response to ATP depletion.

Cells were isolated from normoglycemic (N) and streptozotocin-injected (55 mg/kg) rats (D) treated or not treated with losartan (20 mg/kg/day in the drinking water; NL and DL, respectively) for 3 weeks. In each group of cells, enzyme activities such as glucose-6-phosphate (G6PDH) and glycerol-3-phosphate dehydrogenases (G3PDH), lactate/pyruvate, glycogen levels and citrate synthase were measured as an index of glycolytic dysregulation and mitochondrial mass, respectively.

Cells were then challenged with NaCN (2 mM) in glucose-free Tyrode solution (metabolic intoxication, MI), a protocol to study ischemia at cell level. Under these conditions, the time to contractile failure up to rigor-type hyper-contracture in field-stimulated cells and K_{ATP} current activation by patch-clamp recordings were measured.

In comparison with N and NL, D cells presented higher G6PDH and cytoplasmic G3PDH activities, lactate/pyruvate, glycogen content but similar levels of citrate synthase, and decay time of contraction. When subjected to MI, D cells showed delayed activation of the K_{ATP} current (25.7 ± 7.1 min; $p < 0.001$ vs. N and NL), increased time to contractile failure and rigor-type hyper-contracture ($p < 0.001$ vs. N and NL). In cells from DL rats both functional (time to rigor and to K_{ATP} current activation) and metabolic parameters, approached values similar to those measured in N and NL cells.

These results demonstrate that diabetic cardiomyocytes from rats treated with losartan, maintain the capacity to respond promptly to ATP depletion reaching contractile failure, rigor-type hypercontracture and K_{ATP} opening with a similar timing of N cells.

1. Introduction

Clinical evidence strongly suggests that hyperglycemia and cardiac ischemic events are detrimentally related [1]. While insulin resistance and diabetes are associated with increased risk of myocardial ischemic disease, undiagnosed diabetes and impaired glucose tolerance are commonly found in patients with acute myocardial infarction [2]. Although the exact mechanisms underpinning this relationship remain poorly understood, it is generally accepted that the metabolic disorder secondary to local insulin resistance can be a culprit in the pathogenesis of diabetic cardiomyopathy that includes increased cardiac susceptibility to ischemia.

In contrast to overwhelming clinical evidence, experimental studies on the sensitivity of the diabetic heart to ischemia are inconsistent [3–5], up to now, it is not clear whether increased risk of cardiovascular events in the diabetic heart is related to intrinsic cardiomyocyte defects or secondary to vascular problems.

Besides obvious limitations [6], *in vitro* or *ex vivo* experimental studies aimed at *studying* cardiomyocyte susceptibility to ischemia offer the advantage of eliminating all the other cell types present in the whole heart. In this context, the use of mitochondrial poisoning, which reduces ATP availability for contraction, mimics the metabolic condition occurring in ischemic myocardium, and is the model to study the timing of recruitment of mechanisms that protect cells under conditions of low energy availability (ischemic and or hypoxic injuries) [6]. In reduced energy supply conditions, glycolytic uncoupling with lactate accumulation and mitochondrial oscillations have been recognized as critical factors complicating the energy supply demand of the heart and producing electrophysiological heterogeneity [7,8].

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doi:10.1016/j.phrs.2010.11.001

Both the depletion of cellular ATP and fall in pH trigger contractile failure and prompt opening of K_{ATP} potassium channels which are fine sensors linking cell energy status to electrogenesis. Their opening reduces cell excitability allowing cells to survive under conditions of low energy availability. Millimolar concentrations of ATP block channel opening, while critically low levels open them, attempting to reduce cell energy expenditure and minimizing calcium loading. In addition, activation of K_{ATP} channels ensures a fully polarized diastolic potential which assists calcium efflux (via the Na^+/Ca^{2+} exchanger), further reducing calcium overload [9]. The occurrence of a K^+ current consisting of "cross-talking" between sarcolemmal and mitochondrial K_{ATP} potassium channels is also crucial to ensuring the protection offered by ischemic preconditioning [10], a feature lost bydiabetic cells [11–13].

Angiotensin-II (AT-II), the principal effector of the renin–angiotensin system, is a hypertrophic independent risk factor for cardiovascular diseases [14]. In the heart, AT-II promotes short-and long-term metabolic and functional changes which include increase of oxidative stress, cardiomyocyte death and contractile dysfunction. AT-II also alters cell levels of modulators of ischemic injury, including activation of PKC [15] and calcium transport [16] and it might play a role in raising insulin-resistance [17]. Moreover, a direct role for AT-II in altering the sensor activity of plasmalemma K_{ATP} in normoglycemic and diabetic cardiomyocytes has been documented [18,19]. Since most of the deleterious cardiovascular effects of AT-II derive from angiotensin type 1 receptor (AT1) activation, the use of AT1 antagonists, represents a first line cardioprotective strategy [20,21].

Losartan is a first generation AT1 antagonist whose clinical effectiveness includes reduction of incidence of cardiac ischemic events in risk populations, including diabetic patients [21,22].

Losartan is a pro-drug which is activated in liver with the production of two metabolites one of which blocks AT1-mediated effects of AT-II, including vascular effects, while the other would be responsible for ancillary features, such as reduction of oxidative stress [23] and reversion of diabetes-induced mitochondrial dysfunction [24]. Up to now, it is not completely established whether the mechanism responsible for protection against ischemia goes beyond losartan hemodynamic features and instead includes direct effects on cardiomyocytes.

In streptozotocin (STZ)-injected rats, a widely used experimental model to study diabetes-related cardiovascular complications [25], we have previously reported that losartan treatment for three weeks ameliorated functional vascular dysfunction [26], insulin resistance, and short ensaction potential duration [27]. In particular, in these latter work we demonstrated that amelioration of insulin resistance correlated with restoration of electrophysiological features. Now, to further understand the spectrum and the mechanism of the cardiovascular protection afforded by losartan in this animal model, we decided to study the effects of drug treatment on diabetic cardiomyocyte abnormal glucose metabolism and their behaviour at condition of reduced cell ATP following metabolic intoxication (MI), a method to study ischemia at cell level.

We isolated cardiomyocytes from normoglycemic and STZ-injected rats (2-weeks after injection) with and without treatment with losartan [27]. In these cells, we measured enzyme activities indicative of glycolytic oscillation (glucose-6-phosphate, G6PDH, soluble glycerol-3-phosphate dehydrogenases, G3PDH, lactate/pyruvate and the glycogen content), mitochondrial proteins as index of mitochondrial mass (citrate dehydrogenase), and cell shortening features. Cells were then perfused with cyanide (MI), an inhibitor of complex IV of the mitochondrial respiratory chain. In this condition, the time of occurrence of the K_{ATP} current and the time the cells spent to reach rigor-type contracture were measured.

2. Materials and methods

2.1. Experimental design

All of the experiments were carried out in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) for experimental animal care.

Wistar rats aged 12–14 weeks (Charles River, Calco Italy) were randomly divided into four groups (see below for description) and allowed free access to standard dried chow diet and water whose consumption was monitored daily.

Two groups of rats were assigned to receive losartan in the drinking water (20 mg/kg/day) until the end of the experiment (3 weeks afterward). After 1 week, two groups, one of the two receiving losartan, were injected in the tail vein with citrate buffer pH 4.5 (normoglycemic, N and normoglycemic, losartan-treated, NL). The other two groups received a single injection of STZ (50 mg/kg in citrate buffer pH 4.5). We checked plasma glycemia of rats for 48 h following STZ injection and only those rats whose glycemia was higher than 14 mM were considered diabetic and included in our schedule of treatment (diabetic, D and diabetic, losartan-treated, DL). The losartan concentration was adjusted according to body weight and water consumption to maintain a dosage of 20 mg/kg/day and the treatment was interrupted 24 h before sacrificing.

2.2. Cell isolation

Left ventricular myocytes were isolated from each group of rats using a protocol based on previously described procedures [28]. Rats were injected with 500 IU heparin ip, anesthetized with ether, and decapitated. After thoracotomy, each heart was rapidly excised, weighed, mounted in a Langendorff apparatus, and perfused for 20 min with low-calcium solution (LCS) (see "Section 2.9") pre-warmed to 37 °C, and equilibrated with 100% O_2. The solution was then quickly changed to LCS plus 1 mg/ml collagenase (Type I, Worthington Biochemical, Lakewood, NJ, USA), 0.03 mg/ml dispase (Boehringer, Bergamo, Italy), 1 mg/ml albumin (Fatty Acid Free Fraction V, Sigma, St. Louis, LO, USA) for 5–10 min. The left ventricle and septum were removed, minced and stirred in the LCS. Cardiomyocytes appearing in the supernatant were purified by gravity sedimentation, collected and stored at room temperature in LCS supplemented with 0.5 mM $CaCl_2$ and 4% penicillin/streptomycin (Gibco BRL, Milan, Italy) and used within the day.

2.3. Enzyme assays

For enzyme determinations, cardiomyocytes were homogenized in ice-cold phosphate buffer (0.1 M; pH 7.4) containing sucrose (0.25 M; 10 mg cell pellet/500 μl buffer). The homogenate was centrifuged ($1000 \times g \times 10$ min at 4 °C) to remove cell debris. The resulting supernatant was subjected to a second centrifugation ($15{,}000 \times g \times 30$ min at 4 °C). The pellet containing mitochondria was washed twice by re-suspension and subsequent centrifugation in the same phosphate buffer containing sucrose. Dried mitochondria were stored at −80 °C until they were used. The $15{,}000 \times g$ supernatant was used for the determination of the soluble enzyme activities.

The protein concentration of the $15{,}000 \times g$ supernatant and of its related pellet (mitochondrial fraction) was determined by the BCA protein assay reagent kit (Pierce) before being frozen at −80 °C.

G6PDH activity was measured in the $15{,}000 \times g$ supernatant by the increase in optical density (OD) at 340 nm due to the conversion of NAD to NADH [29]. Briefly, 50 μl aliquots of the supernatant were incubated in Tris–HCl (50 mM) pH 8.1 containing $MgCl_2$ (1 mM) and NADP (0.1 mM). The reaction was started by the addition of