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Enhanced oxidative stress sensitizes the mitochondrial permeability transition pore to opening in heart from Zucker Fa/fa rats with type 2 diabetes

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ABSTRACT

Aims: Obesity and diabetes mellitus type 2 (DM2) frequently coexist and increase the propensity of cardiovascular dysfunction by numerous mechanisms. Chief among them are oxidative stress and Ca^{2+} dysregulation, and both are inducers of the mitochondrial permeability transition pore (MPTP). Nevertheless, it is unknown whether MPTP formation is triggered in DM2 animals, and thereby contributing to cardiac dysfunction. We assessed MPTP sensitivity and reactive oxygen species production in cardiac mitochondria, as well as cytosolic Ca^{2+} handling in ventricular myocytes from rats with DM2.
Main methods: Male Zucker Fa/fa rats (Fa/fa) 32 weeks old presenting DM2, concentric hypertrophy, and diastolic dysfunction were used. MPTP formation was evaluated in isolated mitochondria and Ca^{2+} handling in ventricular myocytes, by spectrophotometric and confocal microscope techniques, respectively.
Key findings: We found that the systolic Ca^{2+} transient relaxation was ~40% slower, while mitochondrial H_2O_2 production increased by ~6-fold. MPTP opening in isolated mitochondria from Fa/fa (mFa/fa) was more sensitive to Ca^{2+} than in mitochondria from lean rats (mLean), and correlated with increased thiol group exposure. The mFa/fa showed decreased oxidative phosphorylation capacity. The ATP content decreased in myocytes, while the PCr/ATP ratio remained unchanged and caspase 9 activity largely increased in myocytes from Fa/fa animals.
Significance: Our results showed that oxidative stress and diastolic Ca^{2+} dysregulation increased MPTP sensitivity leading to mitochondrial dysfunction and apoptosis. Mitochondrial dysfunction could compromise ATP synthesis, and lower ATP could be linked to decreased SERCA2 activity, which might underlie diastolic dysfunction. Prolonged Ca^{2+} transients might further exacerbate mitochondrial dysfunction.

1. Introduction

Obesity is a chronic disease characterized by excessive accumulation of fat that underlie multiple metabolic disorders such as hyperlipidemia, hypercholesterolemia, and most commonly, diabetes mellitus type 2 (DM2), and all those disorders frequently coexist leading to cardiovascular diseases [14]. Among the cardiac alterations induced by these metabolic disorders are the following: left ventricular (LV) hypertrophy, chronic volume overload, systolic and diastolic dysfunction, coronary artery disease and heart failure [34]. Several mechanisms have been described to explain the impact of metabolic disorders induced by excessive caloric intake on the development of heart dysfunction. These include: i) endothelial dysfunction [67], ii) high intracellular lipid accumulation, as observed in hearts from obese Zucker Fa/fa rats, due to an increase in long-chain fatty acids transport across the sarcolemma [30]; iii) maladaptive cardiac remodeling, leading to diastolic dysfunction and progression toward systolic dysfunction, mainly due to hypertrophy and pronounced increase in LV-fibrosis [39]; iv) activation of the NADPH oxidase, which induces oxidative damage to proteins, including those responsible of Ca^{2+}-cycling, such as SERCA2, and thereby altering cellular Ca^{2+} homeostasis [36]; and v) cardiac muscle cell loss due to enhanced apoptosis [4,40].

Mitochondrial dysfunction could be a key component of some of the mechanisms underlying cardiac dysfunction due to the metabolic disorders induced by obesity. This is because, in addition to the important role of mitochondria in energy production, they are the most important generators of reactive oxygen species (ROS) [58], particularly upon increased availability of fatty acids [66], and excessive ROS are particularly disruptive of normal cellular functions [71]. Furthermore, mitochondria

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are important participants in cell death regulation, since the release of cytochrome c and other proapoptotic proteins is involved in the intrinsic apoptotic pathway [9,52].

One of the phenomena associated with mitochondrial dysfunction is the change in the mitochondrial membrane permeability due to the formation of a nonspecific pore (mitochondrial permeability transition pore; MPTP), which allows passage of ions and <1500-Da molecules. However, neither the molecular identity nor the physiological function of the MPTP has been completely elucidated. The most commonly accepted theory about the molecular identity is that the MPTP is constituted by mitochondrial proteins that under physiological conditions exert specific normal functions, such as the adenine nucleotide translocase (ANT), the voltage-dependent anionic channel (VDAC) and cyclophilin D [2]. More recently, other proteins have been involved in MPTP formation; e.g., the phosphate carrier [72] and the mitochondrial ATP synthase [27]. Other proteins such as Bax, the mitochondrial benzodiazepines receptor, and the hexokinase have also been associated with the MPTP formation [12,68].

In pathological conditions, the MPTP opening can be induced by a large number of factors, however, the most important are: high cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) [62] and high ROS [56]. The proapototic protein Bax is another important inducer that interacts with the MPTP components allowing the release of cytochrome c to the cytosol triggering apoptosis [17,45]. Obesity, and its comorbidities such as DM2, exacerbates the three factors that determine MPTP opening: i) it slows Ca^{2+} transient relaxation in obese Zucker Fa/fa rats [31,32] and increases resting $[Ca^{2+}]_i$ in hearts from ob/ob mice (leptin-deficient) [36], due to decreased SERCA2 activity or protein content. Slower cytosolic Ca^{2+} removal could potentially enhance Ca^{2+} uptake into the mitochondrial matrix by the Ca^{2+} uniporter (MCU) [6]; ii) it increases NADPH oxidase-dependent ROS production in humans [22]; and iii) in the fat Zucker Fa/fa rats, Bax expression is increased [4,40]. In addition, the presence of cytochrome c in the cytosolic fraction of hearts from obese patients and in Zucker Fa/fa rats has also been reported [51].

Nonetheless, there are no reports demonstrating that MPTP formation is a crucial event in the mechanism involved in the activation of cell death due to the metabolic disorders accompanying obesity, and this could be a major factor underlying cardiac dysfunction. In this regard, a recent report suggested that leptin triggers apoptosis because it may function as an MPTP inducer through the activation of the leptin receptor and the JAK/STAT signaling cascade [46]. Since the Zucker Fa/fa rats have a mutation in the leptin receptor, making them insensitive to leptin, they are a model for obesity, metabolic syndrome and DM2 (depending on the food type and age), where the MPTP formation, independent of the JAK/STAT signaling cascade, can be assessed. Therefore, we sought to determine whether in 32 weeks old Zucker Fa/fa rats, with DM2 signs and cardiac dysfunction, have impaired mitochondrial homeostasis; reflected as diminished structural stability due to MPTP opening and decreased energy production. To this end, we assessed MPTP sensitivity in isolated mitochondria from Zucker Fa/fa rats. We also assessed whether mitochondrial ROS production and cellular Ca^{2+} handling were altered, since both could affect MPTP sensitivity, undermine mitochondrial integrity, decreasing energy production, and ultimately leading to cell death.

2. Materials and methods

2.1. Ethical animal handling

All experiments were performed in accordance with the animal care guidelines of the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1996). All procedures were approved by the animal use and care committee of the School of Medicine, Tecnológico de Monterrey (Protocol # 2012–003).

2.2. Animal model

Male lean (n = 25, 400–450 g) and Zucker Fa/fa (n = 25, 750–820 g), 32-week-old, rats were used. Rats were identified and selected at 4 weeks of age by analysis of the leptin receptor gene mutation. All animals were kept in standard husbandry conditions, fed with regular Purina rat chow ad libitum and had free access to water. To begin the studies, animals were anesthetized with sodium pentobarbital (40 mg/kg) for echocardiographic studies. The following day the rats were euthanized with a sodium pentobarbital overdose (60 mg/kg). Afterwards, a sample of blood for biochemical analysis was collected from the abdominal aorta, and the heart was rapidly excised and placed in cold buffer with the following composition (in mM): 250 sucrose, 10 HEPES and 1 EGTA, pH 7.3, at 4°°C.

2.3. Echocardiography

Echocardiographic studies were carried out using a Philips EnVisor HD with a transducer of 5–12 MHz. Data images were analyzed with OsiriX software, 32-bit version 5.0.2. For these studies the anesthetized rats were placed in the left lateral decubitus position. The probe was positioned to obtain M-Mode, parasternal long and short axis view, four chambers and five chambers view. M-Mode was used to measure left ventricular anterior wall and interventricular septal wall thickness. The parasternal short axis and papillary muscle level were also assessed. Aortic flow was assessed from the 4-chamber apical view by using pulsed-wave Doppler. All parameters were averaged over at least five cardiac contractile cycles per animal. Fractional shortening was calculated by the equation: [left ventricular diameter in diastole − left ventricular diameter in systole / left ventricle diameter in diastole] × 100. Volumes were calculated by hemi-ellipse formula. Telesystolic volume: [(5/6 × left ventricle epicardium area in systole) × (length of left ventricle in systole)], telediastolic volume: [(5/6 × left ventricle epicardium area in diastole) × (length of left ventricle in diastole)]. Ejection fraction was calculated using the formula: ((telediastolic volume − telesystolic volume) / (telediastolic volume)).

2.4. Biochemical analyses

Plasma leptin was assessed by ELISA in 10 μl of plasma using a commercially available kit (Murine Leptin ELISA development kit, Peprotech®) following the manufacturer's specifications. Results are reported in nanograms per milliliter (ng/ml). Plasma triglycerides and glucose were quantified in 5 μl of plasma using a commercially available kit (Triglycerides Liquicolor® GPO-PAP, Stanbio; and Glucose colorimetric assay kit, Cayman Chemical, 10,009,582, respectively). Plasma HDL, LDL and cholesterol were quantified in 100 μl of plasma (Assay kit, Abcam ab65390), according to the manufacturer's specifications. Results are reported in milligrams per deciliter (mg/dl).

2.5. Ventricular myocytes isolation

Myocytes were dissociated as previously described [18]. Rats were euthanized as described above. Excised hearts were mounted on a Langendorff system and perfused with collagenase-containing solution (1 mg/ml collagenase type II; Worthington Biochemical Corporation, Lakewood, NJ, USA) at 37°°C. Upon digestion, ventricles were mechanically disaggregated, and dissociated cells were kept at room temperature in normal Tyrode's (NT) solution, containing (in mM): 140 NaCl, 4 KCl, 1 $MgCl_2$, 10 HEPES, 10 glucose, and 1 $CaCl_2$, pH 7.4 adjusted with NaOH.

2.6. Cellular Ca^{2+} signaling

Ventricular myocytes were incubated with Fluo-4 AM (10 μM in NT; Life Technologies, Carlsbad, CA, USA) for 30 min. Thereafter, excess