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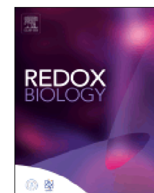

Angiotensin receptor blockade improves cardiac mitochondrial activity in response to an acute glucose load in obese insulin resistant rats

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ABSTRACT

Hyperglycemia increases the risk of oxidant overproduction in the heart through activation of a multitude of pathways. Oxidation of mitochondrial enzymes may impair their function resulting in accumulation of intermediates and reverse electron transfer, contributing to mitochondrial dysfunction. Furthermore, the renin-angiotensin system (RAS) becomes inappropriately activated during metabolic syndrome, increasing oxidant production. To combat excess oxidant production, the transcription factor, nuclear factor erythriod-2- related factor 2 (Nrf2), induces expression of many antioxidant genes. We hypothesized that angiotensin II receptor type 1 (AT1) blockade improves mitochondrial function in response to an acute glucose load via upregulation of Nrf2. To address this hypothesis, an oral glucose challenge was performed in three groups prior to dissection (n = 5–8 animals/group/time point) of adult male rats: 1) Long Evans Tokushima Otsuka (LETO; lean strain-control), 2) insulin resistant, obese Otsuka Long Evans Tokushima Fatty (OLETF), and 3) OLETF + angiotensin receptor blocker (ARB; 10 mg olmesartan/kg/d × 6 weeks). Hearts were collected at T0, T60, and T120 minutes post-glucose infusion. ARB increased Nrf2 binding 32% compared to OLETF at T60. Total superoxide dismutase (SOD) and catalase (CAT) activities were increased 45% and 66% respectively in ARB treated animals compared to OLETF. Mitochondrial enzyme activities of aconitase, complex I, and complex II increased by 135%, 33% and 66%, respectively in ARB compared to OLETF. These data demonstrate the protective effects of AT1 blockade on mitochondrial function during the manifestation of insulin resistance suggesting that the inappropriate activation of AT1 during insulin resistance may impair Nrf2 translocation and subsequent antioxidant activities and mitochondrial function.

1. Introduction

Metabolic syndrome is a rising epidemic in the western world and is characterized by the simultaneous presence of hypertension, dyslipidemia, elevated fasting plasma glucose levels, abdominal obesity, and insulin resistance [11]. Insulin resistance (IR) is a hallmark for the progression of type II diabetes and causes an incomplete uptake of circulating plasma glucose due to impaired insulin secretion and/or receptor signaling [1]. Inappropriate activation of the renin-angiotensin system (RAS) through the angiotensin II type I (AT1) receptor occurs during insulin resistance and has been implicated in contributing to cardiovascular derangements not limited to vasoconstriction, thrombosis, and cardiovascular remodeling [9]. Mitochondrial dysfunction contributes to heart disease, and may contribute disproportionately to the accumulation of oxidative damage during diabetes [2,19]. Furthermore, cardiomyocytes contain larger amounts of mitochondria compared to other tissues [27], while the heart as a whole contains a lower antioxidant capacity [33] which increases its susceptibility to mitochondria-derived oxidation. In turn, mitochondrial oxidation increases oxidant generation further burdening antioxidant enzymes, which may lessen their ability to correct the oxidant imbalance [14]. Furthermore, mitochondrial and antioxidant dysfunctions may be exacerbated by post-prandial glucose mediated oxidant production in insulin resistant individuals [3,34]. Among the enzymes that are altered by oxidized conditions are aconitase, and in the citric acid cycle, NADH dehydrogenase (complex I), succinate dehydrogenase (complex II), and cytochrome c reductase (complex III) [4,22].

Cellular detoxification is an important process that helps remove excess oxidants from the cellular environment through endogenous antioxidants or other molecules capable of reduction. One of the key

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regulators of antioxidant production is the transcription factor, nuclear factor (erythroid-derived 2)-like 2 (Nrf2), which regulates the production of genes responsible for cellular detoxification among other functions [13]. Nrf2 controls production of several antioxidants such as catalase (CAT), glutathione peroxidase (GPx), and superoxide dismutase (SOD), which together aid in neutralizing excess oxidant production [35]. In order for Nrf2 to translocate to the nucleus, it must dissociate from Kelch-like ECH-associated protein 1 (Keap1) in the cytosol usually by oxidation of Keap1's thiols [7].

The impacts of insulin resistance on the acute changes that glucose imposes on cellular metabolism in the heart are not well described. Evaluation of the heart's mitochondrial response to an acute challenge provides insight on its adaptability and potential to recover from such a constant and frequent insult. This study provides novel information on how cardiac mitochondria respond to a large influx of glucose within two hours post ingestion and the effects of angiotensin receptor blockade (ARB) treatment on the insulin resisitant heart. We hypothesized that AT1 receptor blockade improves cardiac mitochondrial function in response to a glucose load via upregulation of Nrf2.

2. Methods

All experimental procedures were reviewed and approved by the institutional animal care and use committees of Kagawa Medical University (Kagawa, Japan), and the University of California, Merced.

2.1. Animals

Male, age matched, 10-week-old, lean strain-control Long Evans Tokushima Otsuka (LETO; 279 ± 7 g) and obese Otsuka Long Evans Tokushima Fatty (OLETF; 359 ± 3 g) rats (Japan SLC Inc., Hamamatsu, Japan) were chosen because OLETF rats were previously shown to be insulin resistant at the timeframe chosen for this study [24]. LETO and OLETF rats were assigned to the following groups (n = 5–8 animals/group/time point): 1) untreated LETO, 2) untreated OLETF, and 3) OLETF + angiotensin receptor blocker (ARB; 10 mg olmesartan/kg/d × 6 wk). ARB (Daiichi-Sankyo, Tokyo, Japan) was administered by oral gavage suspended in carboxymethyl cellulose (CMC) to conscious rats. Untreated LETO and OLETF rats were gavaged with CMC only. All animals were maintained in groups of three or four animals per cage in a specific pathogen-free facility under controlled temperature (23 C) and humidity (55%) with a 12-h light, 12-h dark cycle. All animals were given free access to water and standard laboratory rat chow (MF; Oriental Yeast Corp., Tokyo, Japan).

2.2. Body mass (BM)

BM was measured on a daily basis to calculate the appropriate amount of ARB to gavage.

2.3. Blood pressure

Systolic blood pressure (SBP) was measured at 16 weeks of age in conscious rats by tail-cuff plethysmography (BP-98A; Softron Co., Tokyo, Japan).

2.4. Dissections

After 6 weeks of ARB treatment, animals were randomly assigned to 3 different subgroups within each group. Following the subgroup assignment animals were fasted overnight (12 h). The first subgroup of animals was taken following the overnight fast (T0), the second and third subgroups of animals were taken 1 h (T60) and 2 h (T120), following a glucose load (2 g/kg). This protocol allowed us to ascertain the cellular events that transpired in the heart for 2 h post-glucose load. At dissection, animals were decapitated, and trunk blood was collected into chilled vials containing 50 mM EDTA and protease inhibitor cocktail (sigma), and kept on ice until they could be centrifuged. Thereafter the hearts were rapidly removed, weighed, and snap frozen in liquid nitrogen. Frozen samples were kept at −80 C until analyzed.

2.5. Western blot analyses

A 35 mg piece of frozen heart was homogenized in 250 µl of Tris-buffered saline containing Triton X-100, SDS, and protease and phosphatase inhibitor cocktail (Sigma). Tissue homogenate was centrifuged (13,200 × *g*, 10 min), and the aqueous layer was transferred to a separate tube and stored at −80 C for later analyses. Membrane fractions were obtained for p47phox translocation using a Minute Plasma Membrane Protein Isolation Kit (Invent Biotechnologies, Plymouth, MN). Membrane fractions were cross-probed with Na^+/K^+ ATPase and GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA) antibodies to ensure fraction purity. Total protein content was measured by the Bradford assay (Bio-Rad Laboratories, Hercules, CA). Equal loading of five to forty micrograms of total protein were resolved in 4–15% Tris-HCl SDS gradient gels. Proteins were electroblotted by 2 h wet transfer onto 0.45-µm polyvinyl difluoride membranes. Membranes were blocked with LI-COR Odyssey blocking buffer and incubated for 16 h with primary antibodies (diluted 1:200 to 1:2000) against Keap1 (Santa Cruz Biotechnology, Santa Cruz, CA), AMPK, phosphorylated (p) AMPK at Thr172, Nitrotyrosine (Cell Signaling, Danvers, MA), MnSOD, Cu/ZnSOD (Stressgen, Farmingdale, NY), P47phox and 4HNE (Milipore, Bedford, MA). Membranes were washed, incubated with IRDye 800CW and/or 700CW donkey anti-goat, donkey anti-mouse, or donkey anti-rabbit (LI-COR Biosciences, Lincoln, NE), and rewashed. Blots were visualized using an Odyssey system (LI-COR Biosciences) and quantified using ImageJ. In addition to consistently loading the same amount of total protein per well, the densitometry values were further normalized by correcting with the densitometry values of Ponceau S staining [28].

2.6. Biochemical analyses

Nrf2 (Active Motif, Carlsbad, CA), antioxidant enzymes and (CAT, GPx, and SOD) and aconitase (Cayman Chemical, Ann Arbor, MI) activities, and other mitochondrial activities (Succinate Dehydrogenase, and NADH Dehydrogenase) (Abcam, Cambridge, MA) were measured using commercially available kits as previously described [32]. Nrf2 binding was measured using this kit to best assess its binding to the electrophile response element (EpRE). Nuclear fractions were prepared for Nrf2 activity using a NE-PER Nuclear cytosolic extraction kit (Thermo Fisher Scientific, Waltham, MA). Nuclear purity was cross probed with GAPDH and H3 (Cell Signaling, Danvers, MA). Mitochondrial fractions were obtained according to the Aconitase assay instructions. Mitochondrial purity was cross probed using GAPDH and VDAC1 (Abcam, Cambridge, MA). All samples were analyzed in duplicate and run in a single assay with intra-assay and percent coefficients of variability of less than 10% for all assays.

2.7. Statistics

Means (± SEM) were compared by two-way ANOVA adjusted for repeated measures to group and time interactions. Pairwise comparisons were made for individual time points. Means were considered significantly different at $P < 0.05$ using Fisher's PLSD. Statistical analyses were performed with the SPSS version 24 software (IBM, Armonk, NY).

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