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Original article

Antioxidant treatment normalizes mitochondrial energetics and myocardial insulin sensitivity independently of changes in systemic metabolic homeostasis in a mouse model of the metabolic syndrome

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ABSTRACT

Cardiac dysfunction in obesity is associated with mitochondrial dysfunction, oxidative stress and altered insulin sensitivity. Whether oxidative stress directly contributes to myocardial insulin resistance remains to be determined. This study tested the hypothesis that ROS scavenging will improve mitochondrial function and insulin sensitivity in the hearts of rodent models with varying degrees of insulin resistance and hyperglycemia. The catalytic antioxidant MnTBAP was administered to the uncoupling protein-diphtheria toxin A (UCP-DTA) mouse model of insulin resistance (IR) and obesity, at early and late time points in the evolution of IR, and to db/db mice with severe obesity and type-two diabetes. Mitochondrial function was measured in saponin-permeabilized cardiac fibers. Aconitase activity and hydrogen peroxide emission were measured in isolated mitochondria. Insulin-stimulated glucose oxidation, glycolysis and fatty acid oxidation rates were measured in isolated working hearts, and 2-deoxyglucose uptake was measured in isolated cardiomyocytes. Four weeks of MnTBAP attenuated glucose intolerance in 13-week-old UCP-DTA mice but was without effect in 24-week-old UCP-DTA mice and in db/db mice. Despite the absence of improvement in the systemic metabolic milieu, MnTBAP reversed cardiac mitochondrial oxidative stress and improved mitochondrial bioenergetics by increasing ATP generation and reducing mitochondrial uncoupling in all models. MnTBAP also improved myocardial insulin mediated glucose metabolism in 13 and 24-week-old UCP-DTA mice. Pharmacological ROS scavenging improves myocardial energy metabolism and insulin responsiveness in obesity and type 2 diabetes *via* direct effects that might be independent of changes in systemic metabolism.

1. Introduction

Type-two diabetes and the metabolic syndrome more than double the risk for cardiovascular disease (CVD), a leading cause of death in middle- and high income countries [1]. Therapeutic strategies based on lowering circulating glucose levels, have not reversed major cardiovascular complications in multiple clinical trials [2,3], emphasizing that organ-specific mechanisms may play a significant role in the pathogenesis of CVD in insulin resistant subjects. The cardiomyocyte-specific changes may include decreased insulin-stimulated glucose uptake and utilization, mitochondrial dysfunction and oxidative stress. Several lines of evidence suggest that insulin resistance is associated with mitochondrial abnormalities in the heart. Earlier studies in animal models have shown that in the hearts of diabetic and obese db/db and ob/ob mice, insulin resistance was observed together with impaired mitochondrial function, and increased production of reactive oxygen species (ROS) [4–6]. Consistent with these observations, genetic disruption of insulin signaling or insulin-stimulated glucose utilization in cardiomyocytes, promotes oxidative stress and mitochondrial dysfunction [7,8]. *In vitro*, TNFα, dexamethasone or palmitate treatments impaired insulin-stimulated glucose transport and induced oxidative stress in cultured 3 T3-L1 adipocytes or L6 myotubes [9,10].

Although oxidative stress and mitochondrial dysfunction accompany the insulin resistant cardiac phenotype, it is incompletely understood whether insulin resistance is primary or secondary to oxidative stress or

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mitochondrial dysfunction in the heart. This study was designed to test the hypothesis that myocardial insulin-resistance in obesity is secondary to mitochondrial dysfunction and increased oxidative stress. If true, then treatment with a mitochondria-targeted antioxidant might restore mitochondrial function and improve myocardial insulin sensitivity. To test this hypothesis, we used several mouse models of mild to severe hyperglycemia and obesity, namely UCP-DTA and db/db mice [5,11]. UCP-DTA mice have a defect in peripheral energy expenditure and develop obesity on normal chow diet. Their pathophysiology has been extensively studied and parallel many of the metabolic abnormalities that are seen in animals fed a high-fat diet [12]. Moreover, the fatty acid composition of certain high-fat diets might have independent mitochondrial effects, which could confound the mitochondrial phenotype [13–15]. db/db mice were chosen to model extreme insulin resistance, obesity and diabetes. Importantly, both of these models have been previously characterized and shown to have impaired myocardial mitochondrial ATP synthesis [5,11].

In addition to being the primary source of energy in the heart, mitochondria are an important source of superoxide that may contribute to oxidative stress. Therefore, we chose to pharmacologically inhibit ROS in the heart by utilizing catalytic activity of the mitochondrial antioxidant Mn (III) tetrakis (4-benzoic acid) porphyrin (MnTBAP), an established pharmacological scavenger of superoxide, whose mitochondrial superoxide dismutase (SOD2 or MnSOD) and catalase activity helps to detoxify superoxide to water [16,17]. Treatment of ob/ob mice with MnTBAP increased glucose tolerance in the fed state [10], and glucose disposal into muscle and fat in high fat-fed mice [9]. *In vitro*, MnTBAP also restored GLUT4 translocation in insulin resistant L6 myotubes [9].

In the present study we show that systemic administration of MnTBAP reduced cardiac oxidative stress and improved mitochondrial function and myocardial insulin sensitivity in UCP-DTA mice and normalized myocardial mitochondrial dysfunction and fatty acid utilization in db/db mice independent of changes in systemic metabolic homeostasis. Thus, in independent murine models that span the spectrum of obesity and insulin resistance, we provide evidence that targeted antioxidants might act directly to restore mitochondrial bioenergetics and insulin sensitivity in the heart.

2. Methods

2.1. Animals

Protocols for animal care and experimentation were approved by the Institutional Animal Care and Use Committee of the University of Utah. UCP-DTA and littermate control mice (WT) were bred and maintained at the University of Utah until the ages of 13 or 24 weeks. UCP-DTA mice are on the FVB genetic background. Db/db mice and their WT controls were obtained from The Jackson Laboratory and maintained until 9 weeks of age. Db/db mice are on the C57BL/6 J genetic background. All mice were kept in temperature-controlled animal facilities (21–23 °C) on 12 h light (6:00 AM–6:00 PM) and 12 h dark cycle and fed standard chow diet (#8656 Harlan Teklad, Madison, WI) *ad libitum*. MnTBAP [Mn (III) tetrakis (4-benzoic acid) porphyrin chloride solution in alkalized saline (VWR, PA) was administered intraperitoneally to UCP-DTA and wildtype littermate controls (WT) at 20 mg/kg, 3 times/week for 4 weeks starting at the age of 9 or 20 weeks. For db/db mice, MnTBAP was administered 3 times/week for 3–4 weeks starting at the age of 6 weeks.

2.2. Body composition

Mice were sedated with an intraperitoneal (I.P.) injection of chloral hydrate (0.03 mg/g of body weight). Dual Energy X-ray Absorptiometry (DEXA) scans were performed using the Norland pDEXA™ (Trumbull, CT) scanner. In 13 week-old UCP-DTA mice, epididymal and retroperitoneal white adipose tissue was removed and individually weighed.

2.3. Glucose tolerance test

13 and 24 week-old UCP-DTA and 9 week-old db/db mice and respective controls were fasted for 6 hours, and glucose tolerance tests were performed as previously described [18]. Briefly, basal blood glucose levels were measured after a 6-hour fast using a standard glucometer (Bayer, IN). Glucose was dissolved in 0.9% saline (1 mg/g body weight) and administered as a single intraperitoneal injection. Blood glucose was measured right before the injection and 5, 15, 30, 60, and 120 min after the injection using the same glucometer.

2.4. Mitochondrial isolation

Cardiac ventricles were cleaned of blood and minced in STE1 buffer (on ice), and homogenized in a Potter-Elvejhem glass homogenizer. Mitochondria were isolated using a series of differential centrifugations as previously described [7].

2.5. Hydrogen peroxide emission by isolated mitochondria

An increase in the fluorescence of oxidized homovanillic acid by H_2O_2 emitted by the isolated mitochondria was measured as previously described [5]. Briefly, an increase in fluorescence (Ex. 312 nm, Em. 420 nm) was monitored after addition of oligomycin (1 μg/mL) and succinate (4 mM) to isolated cardiac mitochondria. The contribution of complex I to ROS production was assessed following the addition of the complex I inhibitor, rotenone (4 μM).

2.6. Aconitase activity assay

Activity of mitochondrial aconitase was measured in isolated mitochondria as previously described [19].

2.7. Mitochondrial oxygen consumption and ATP production

Mitochondrial respiration rate and ATP synthesis rate were measured as previously described [4,5]. Briefly, basal, ADP-stimulated, oligomycin-inhibited mitochondrial respiration was measured in saponin-permeabilized fibers from left ventricle, using palmitoylcarnitine (0.02 mM) and malate (2 mM) or glutamate (5 mM) and malate (2 mM) as substrates. ATP production was measured in ADP-stimulated samples using the Enliten Luciferase/Luciferin Reagent (Promega, Madison, WI). In addition, db/db hearts were perfused with palmitate (1 mM) and glucose (11 mM) in the Langendorff mode prior to fiber separation and measurement of mitochondrial respiration [5].

2.8. Electron microscopy and stereology

Hearts were removed and 1 mm^3 excised from left ventricular endocardium and subendocardium, and placed in standard fixative (1% paraformaldehyde, 2.5% glutaraldehyde, 0.1 M sodium cacodylate, 2.4% sucrose and 8 mM $CaCl_2$, pH 7.4), processed, and analyzed as previously described [5]. Mitochondrial number and volume density were quantified using stereology.

2.9. Insulin stimulation of mice

Mice were fasted for 6 h and anesthetized with 0.07 mg/g body weight I.P. chloral hydrate. 0.1 IU of human regular insulin or equal volume of saline was injected with via the inferior vena cava (IVC) as previously described [20]. Hearts were then harvested 5 min following injection, and frozen in liquid nitrogen. Cardiac perfusions in the