Original article

MOLECULAR METABOLISM

Glucose-6-phosphate dehydrogenase contributes to the regulation of glucose uptake in skeletal muscle

Robert S. Lee-Young [1,*], Nolan J. Hoffman [4,9], Kate T. Murphy [5], Darren C. Henstridge [1], Dorit Samocha-Bonet [4], Andrew L. Siebel [3,10], Peter Iliades [1], Borivoj Zivanovic [1], Yet H. Hong [6], Timothy D. Colgan [2], Michael J. Kraakman [1], Clinton R. Bruce [7], Paul Gregorevic [2], Glenn K. McConell [6], Gordon S. Lynch [5], Grant R. Drummond [8], Bronwyn A. Kingwell [3], Jerry R. Greenfield [4], Mark A. Febbraio [1,4,]**

ABSTRACT

Objective: The development of skeletal muscle insulin resistance is an early physiological defect, yet the intracellular mechanisms accounting for this metabolic defect remained unresolved. Here, we have examined the role of glucose-6-phosphate dehydrogenase (G6PDH) activity in the pathogenesis of insulin resistance in skeletal muscle.

Methods: Multiple mouse disease states exhibiting insulin resistance and glucose intolerance, as well as obese humans defined as insulin-sensitive, insulin-resistant, or pre-diabetic, were examined.

Results: We identified increased glucose-6-phosphate dehydrogenase (G6PDH) activity as a common intracellular adaptation that occurs in parallel with the induction of insulin resistance in skeletal muscle and is present across animal and human disease states with an underlying pathology of insulin resistance and glucose intolerance. We observed an inverse association between G6PDH activity and nitric oxide synthase (NOS) activity and show that increasing NOS activity via the skeletal muscle specific neuronal (n)NOSμ partially suppresses G6PDH activity in skeletal muscle cells. Furthermore, attenuation of G6PDH activity in skeletal muscle cells via (a) increased nNOSμ/NOS activity, (b) pharmacological G6PDH inhibition, or (c) genetic G6PDH inhibition increases insulin-independent glucose uptake.

Conclusions: We have identified a novel, previously unrecognized role for G6PDH in the regulation of skeletal muscle glucose metabolism.

Keywords Glucose metabolism; Enzyme activity; Insulin sensitivity

1. INTRODUCTION

Skeletal muscle is one of the largest organs in the human body and, quantitatively, the most important tissue involved in maintaining glucose homeostasis under insulin-stimulated conditions [1]. Recently, we demonstrated that skeletal muscle insulin resistance is an early metabolic defect that precedes hyperglycemia and marked weight gain in response to high-fat feeding in mice [2]. While insulin resistance was associated with elevated lipid species we and others have shown a disconnect between these parameters [3,4]. Furthermore, while inflammatory markers have been linked to skeletal muscle insulin resistance [5], gross changes in skeletal muscle inflammation appear to occur well after the induction of insulin resistance [2]. Likewise, in our hands, adipose tissue macrophage accumulation does not affect whole-body insulin action [6]. Thus, other cellular perturbations likely contribute to skeletal muscle insulin resistance.

Another cause of skeletal muscle insulin resistance could be an altered cellular redox state. In cells, the pyridine nucleotide NADPH is required for a number of processes, including maintenance of the cellular redox balance and antioxidant defense [7]. NADPH is required for the

[1]Cellular and Molecular Metabolism Laboratory, Baker IDI Heart & Diabetes Institute, Melbourne, VIC, Australia [2]Muscle Research and Therapeutics Laboratory, Baker IDI Heart & Diabetes Institute, Melbourne, VIC, Australia [3]Metabolic and Vascular Physiology Laboratory, Baker IDI Heart & Diabetes Institute, Melbourne, VIC, Australia [4]Diabetes & Metabolism Division, Garvan Institute of Medical Research, NSW, Australia [5]Basic and Clinical Myology Laboratory, Department of Physiology, The University of Melbourne, Melbourne, VIC, Australia [6]Institute for Sports, Exercise and Active Living, Victoria University, Footscray, VIC, Australia [7]School of Exercise and Nutrition Sciences, Deakin University, Burwood, VIC, Australia [8]Vascular Biology and Immunopharmacology Group, Department of Pharmacology, Monash University, Clayton, VIC, Australia

[9] Current address: Centre for Exercise and Nutrition, Mary MacKillop Institute for Health Research, Australian Catholic University, Melbourne, VIC, Australia.

[10] Current address: School of Biosciences, The University of Melbourne, Melbourne, VIC, Australia.

*Corresponding author. Mouse Metabolic Phenotyping Facility, Metabolic Disease and Obesity Program, Biomedicine Discovery Institute, Monash University, Clayton, VIC, 3800, Australia. E-mail: robert.lee-young@monash.edu (R.S. Lee-Young).

**Corresponding author. Division of Diabetes & Metabolism, Garvan Institute of Medical Research, 384 Victoria St, Darlinghurst, NSW, 2010, Australia. E-mail: m.febbraio@garvan.org.au (M.A. Febbraio).

Received July 29, 2016 • Revision received August 29, 2016 • Accepted September 5, 2016 • Available online xxx

http://dx.doi.org/10.1016/j.molmet.2016.09.002

conversion of oxidized to reduced glutathione (GSSG and GSH, respectively), the primary redox buffer of the cell, which has been shown to be dysregulated in insulin resistant skeletal muscle of rodents and humans [8]. Thus, ensuring adequate cellular NADPH levels is a key requirement for cellular homeostasis. Nevertheless, insulin resistance could also be a by-product of maintaining cellular NADPH levels. Indeed, NAPDH is also the major substrate for NADPH oxidase (Nox), a membrane bound enzyme complex, which generates superoxide ($O_2^{\bullet -}$). Excess $O_2^{\bullet -}$ production has been linked to insulin resistance in skeletal muscle via peroxynitrite ($ONOO^{\bullet -}$) formation [9], and in skeletal muscle the time course of increased Nox expression closely parallels the induction of insulin resistance in response to high-fat feeding [10,11].

In skeletal muscle cells, maintenance of NADPH relies heavily on glucose-6-phosphate dehydrogenase (G6PDH) [12], an enzyme most commonly associated with the pentose phosphate pathway [13]. G6PDH is activated in response to extracellular oxidants that cause a decrease in NADPH levels [14]. Under *in vitro* conditions, it can be regulated by NADPH:$NADP^+$ levels [15]. In diet- and genetic-induced animal models of insulin resistance, G6PDH activity is elevated in adipose tissue [16]. In humans, adipose tissue G6PDH mRNA levels are positively associated with BMI [16], while adenoviral overexpression of G6PDH causes insulin resistance in 3T3-L1 adipocyte cells [16]. Whether G6PDH is mechanistically linked to insulin action in skeletal muscle is unclear.

A potential mechanism linking an altered cellular redox state to insulin resistance is nitric oxide synthase (NOS). In skeletal muscle, the generation of nitric oxide (NO) is regulated by the skeletal muscle specific neuronal NOS isozyme (nNOSμ), which is impaired in insulin resistant states of rodents and humans [17–19]. Similarly, nNOSμ protein expression is almost absent in animal models of muscular dystrophy, and, through the use of this model, it was shown that NO was required to repress G6PDH expression and activity [20]. Thus, it is possible that reduced nNOSμ expression in skeletal muscle of insulin resistant states leads to elevated G6PDH. Alternatively, an increase in $O_2^{\bullet -}$ production – arising from increased Nox – could utilize NO to form $ONOO^{\bullet -}$, which would also act to reduce available NO and lead to increased G6PDH. Collectively, these findings suggest that an altered redox state and/or changes in NO availability (via altered expression of nNOSμ) could be contributing to the onset of skeletal muscle insulin resistance. Thus, we examined whether changes in intramuscular redox state contribute to the induction of insulin resistance in skeletal muscle.

2. MATERIAL AND METHODS

2.1. Animals

C57Bl/6 mice used for the chow-fed and HFD studies have been previously described [2]. C57Bl/10 and *mdx* mice as well as *ob*/+ and *ob*/*ob* littermates were bred in-house (AMREP Animal Services, Melbourne, VIC, Australia). For PBS and C-26 experiments, 21 wk old CD2F1 mice were used as previously described [21]. *nnos*$^{+/+}$ and *nnos*$^{+/-}$ littermate mice were generated by breeding C57Bl/6 nNOS$^{+/-}$ mice originally obtained from Jackson Laboratories (Bar Harbor, ME). All mice were maintained at 22 ± 1 °C on a 12:12 h light–dark cycle with free access to food and water. All procedures undertaken were approved by the AMREP Animal Ethics Committee or the Animal Ethics Committee of The University of Melbourne, and conducted in accordance with the Australian code of practice for the care and use of animals for scientific purposes as stipulated by the National Health and Medical Research Council of Australia.

2.2. Human experiments

Muscle biopsies were collected after an overnight fast from obese insulin sensitive (IS), obese insulin resistant (IT), and pre-diabetic individuals. All protocols were approved by either the Alfred Hospital Human Research Ethics Committee (Melbourne, VIC, Australia) or St. Vincent's Hospital Human Research Ethics Committee (Sydney, NSW, Australia) and conducted in accordance with the Declaration of Helsinki of the World Medical Association. All volunteers provided written informed consent.

2.3. Adenovirus production

Human nNOSμ cDNA was synthesized by GenScript (Piscataway, NJ, USA). Recombinant adenovirus was produced by transfecting HEK293T cells grown to 80–90% confluency. The adenovirus was purified using Mustang QTM ion exchange discs (Pall Corporation, NY, USA) according to manufacturer's instructions. Eluted virus was then concentrated and stored at −80 °C.

2.4. Cell culture experiments

Cell culture experiments were performed on L6 myotubes free of mycoplasma contamination (CRL-1458, ATCC®, USA). For AdV experiments, myotubes were infected with GFP or hu-nNOSμ AdV for 72 h. Glucose transport experiments were performed between passages 2–10 as described [22] using 2-[^{3}H]DG (Perkin Elmer). To determine the effect of G6PDH inhibition on GLUT4 translocation, GLUT4 translocation assays were performed as described on L6 myotubes infected with a retrovirus containing an exofacial HA epitope-tagged construct of human GLUT4 [23]. Stable L6 cells expressing full or partial knockdown of *g6pdh* were generated using a G6PDH shRNA lentivirus in parallel with a scrambled shRNA lentivirus according to the manufacturer's instructions (Santa Cruz Biotechnology Inc.). Where indicated, 6-AN was reconstituted in DMSO (Sigma), and DMSO alone was used as the corresponding control.

2.5. Enzymatic assays

G6PDH activity was measured as the difference between 6-phosphogluconate dehydrogenase (6-PG) activity and total dehydrogenase activity (G6PDH + 6-PG). Samples (~10–20 μg) were incubated in assay buffer (0.1 M Tris–HCl, 500 μM EDTA, 500 μM NADP) with 200 μM 6-phosphogluconate (6-PG activity) or 200 μM G6P + 200 μM 6-phosphogluconate (total activity), and the rate of NADPH production at 340 nm was determined over 20 min (FLUOstar Omega, Life Technologies).

Pyridine nucleotide levels were determined on acid or alkali extracted samples as described [24]. Briefly, ~10–20 μg of protein was added to alkali buffer (0.05 M NaOH, 1 mM EDTA) and then divided into two aliquots. In one of the aliquots, an equal volume of 0.1 M HCl was added to generate an acid extract, and both extracts were then heated at 60 °C for 30 min. The alkali extract was neutralized with 100 mM Tris–HCl (pH 8.1) and 0.05 M HCl. The acid extract was neutralized with 0.4 M Tris. $NADP^+$ and NADPH were measured essentially as described [25] with the exception being that glutamate dehydrogenase and G6PDH, respectively, were used as substrates. The rate of change was measured over 30 min.

NOS activity was determined as described [26] as was GPx activity [27]. GSH(t) and GSSG levels were determined using enzymatic recycling [28]. NADK activity was determined as described and calculated as the difference between samples incubated with and without NAD^+ and ATP.

2.6. RNA isolation and quantitative real-time RT-PCR

Total RNA was isolated from skeletal muscle tissue using Trizol (Invitrogen, Carlsbad, CA). Samples were reverse transcribed using

