Proton NMR metabolomics and genomics show induction of insulin resistance in murine skeletal muscle in response to treatment with a small volatile bacterial molecule

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Introduction– Bacteria excrete small molecules that act as specific chemical signals to positively regulate specialized processes (1). 2-AA is the molecule responsible for the typical grape like odor of P. aeruginosa infected burn wound (2) used in the past as diagnostic tool. However, its effect on the host metabolism and whether it might be involved in insulin resistance remains unknown. Insulin resistance besides being associated with infection, is also frequently observed in various metabolic diseases, including obesity, dyslipidemia, arterial hypertension, and type 2 diabetes. Skeletal muscle wasting in insulin-resistant patients has been documented, and develops due to a lack of anabolic effects, as well as enhanced gluconeogenesis and protein catabolism in skeletal muscle tissue. Although the molecular mechanisms underlying abnormal insulin function has not been elucidated, there is increasing evidence that free fatty acids (FFAs) may play a role in inducing insulin resistance (3). Because fatty acid metabolism and glucose levels are closely linked, aberrations in FFA levels – such as through accumulation of lipids or triglycerides in the muscle – may lead to insulin resistance. It has been suggested that ^{1}H NMR spectroscopy is a useful method for metabolomics, a field which has grown exponentially recently because of improvements in technology. Here, we measured levels of IMCLs visible by High-Resolution Magic Angle Spinning (HRMAS) 1H NMR spectroscopy of gastrocnemius skeletal muscle tissue samples following treatment of mice with 2-AA. We found that increased IMCL levels, a metabolic biomarker for insulin resistance, was associated with altered expression of key regulators of glucose metabolism leading to insulin resistance. Our results enabled us to propose a molecular mechanism governing 2-AA-induced insulin resistance in murine skeletal muscle that may confers mitochondrial dysfunction and a higher risk of systemic nosocomial infection.**Materials and Methods–** Male, 6-wk-old CD1 mice weighing approximately 20-25 g were purchased from Charles River Laboratory (Boston, MA). Mice were treated with 2-AA (500ul 10mM 2AA injected intra-peritoneally) and skeletal muscle samples from the gastrocnemius of mice were collected days following treatment with 2-AA. Animals were studied with HRMAS ^{1}H NMR spectroscopy before and at 6 h, 24 h, and 72 h after burn trauma. Three mice per each category were investigated. HRMAS 1H NMR spectroscopy experiments of muscle tissue were performed on a Bruker Bio-Spin Avance NMR spectrometer (proton frequency at 600.13 MHz, 89 mm Vertical Bore) using a 4-mm triple resonance (1H, ^{13}C, ^{2}H) HRMAS probe (Bruker, Billerica, Massachusetts). The temperature was maintained at 4°C by a BTO-2000 thermocouple unit in combination with a magic angle spinning (MAS) pneumatic unit (Bruker). The MAS speed was stabilized at 4.0 ± 0.001 kHz by a MAS speed controller. The ^{1}H NMR spectra were acquired on all samples using a Carr-Purcell-Meiboom-Gill (CPMG) spin echo pulse sequence, [90°-(t-180°-t)n-acquisition. HRMAS 1H NMR spectra were analyzed using the MestRe-C NMR software package (Mestrelab Research, Santiago de Compostela, Spain, www.mestrec.com). Statistical analysis of HRMAS ^{1}H NMR spectroscopy data. RNA Extraction. At 6 h, 24 h, and 72 h post-burn, 3 burned and 3 control mice were anesthetized by IP injection of 40 mg/kg pentobarbital, and the gastrocnemius was excised and total RNA was extracted. Purified RNA was quantified by UV absorbance at 260 nm and 280 nm and stored at -70°C for DNA microarray analysis. Biotinylated cRNA was generated with 10 µg of total cellular RNA according to the protocol outlined by Affymetrix Inc. (Santa Clara, California). cRNA was hybridized onto MOE430A oligonucleotide arrays (Affymetrix), stained, washed, and scanned according to Affymetrix protocol. The scanned images of cRNA hybridization were converted to cell intensity files (.CEL files) with the Microarray suite 5.0 (MAS, Affymetrix). **Results–** IMCLs as detected by NMR rise in gastrocnemius skeletal muscle of mice treated with 2-AA. Figure 1 shows representative 1H-NMR spectra acquired from normal and 2-AA treated mice, and illustrates a notable rise in IMCLs upon treatment with 2-AA. Treatment with 2-AA changes the expression of genes involved in insulin signaling, glucose transport and other key mitochondrial genes i.e., UCP3 and PGC-1β. In our transcriptome studies, we compared the expression of all genes that might lead to metabolic dysfunction in skeletal muscle after 2-AA treatment. Here, we show the differential expression of 5,055 genes at 4 days following 2-AA treatment (fold change > 2, $p < 0.05$). The differential expression of selected genes is shown in Table 2. We observed downregulation of several genes involved in muscle function (Figure 2).

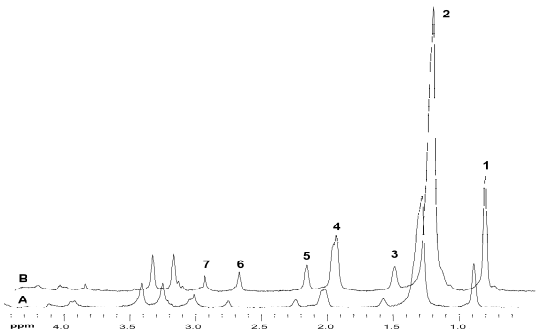

Fig. 1. The spectra were acquired from normal mice and mice at 4 d post-2-AA treatment and scaled to the phosphocreatine and creatine peak (3.02 ppm). Resonance signals of lipids correspond to: 1, terminal methyl CH_3 protons (0.9 ppm); 2, acyl chain methylene protons $(CH_2)_n$ of intramyocellular lipids (IMCLs) (1.3 ppm); 3, β methylene protons CH_2C-CO (1.6 ppm); 4, allylic methylene protons C=C-CH_2-C of monounsaturated fatty acyl moieties (MUFAs) (2.05 ppm); 5, α methylene protons CH_2CO (2.25 ppm); 6, diallylic methylene protons =C-CH_2-C= of polyunsaturated fatty acyl moieties (PUFAs). 7, N-methyl protons of phosphocreatine and creatine (3.0 ppm), respectively.

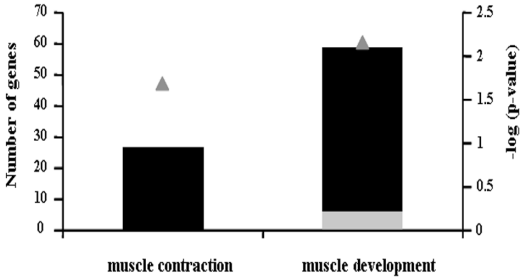

Fig. 2. 2-AA treatment down-regulates muscle function. Black bars indicate the number of down-regulated genes; gray bars indicate the number of up-regulated genes in the skeletal muscle of mice 4 days after 2AA treatment versus control mice (left vertical axis). The negative log10 of p-values represented by gray triangles are indicated in the right vertical axis

Discussion- The present study demonstrates that treatment with a small secreted bacterial molecule, 2-AA, produced by the opportunistic human pathogen Pseudomonas aeruginosa, an "acute insulin resistant state" characterized by: 1) a significant increase of IMCLs or intracellular triglyceride stores in skeletal muscle; 2) a downregulation of IRS1, Glut4, PGC-1β and PPARγ mRNA levels; and 3) an upregulation of UCP3 mRNA levels leading to mitochondrial uncoupling. The strength of HRMAS is that it allows dual investigation of metabolic and molecular changes since the same specimens studied with HRMAS can subsequently be used for transcriptome studies (4). Combining these various results, we propose a potential molecular mechanism for 2-AA-induced skeletal muscle insulin resistance through which a small-excreted bacterial odorant molecule affects glucose transport, insulin signaling and mitochondrial function in skeletal muscle. (Fig. 3). Our results suggest that recent efforts aimed to develop new classes of anti-microbial agents (49) might also lead to the discovery of new host metabolism modulators capable of fighting the deleterious insulin resistant state.

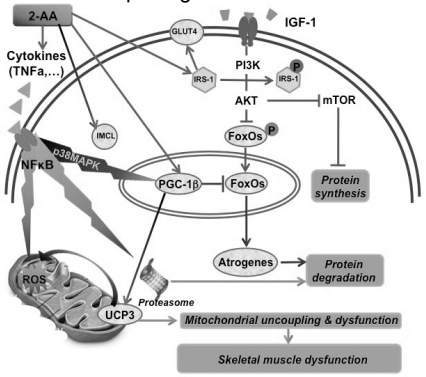

Fig. 3. Proposed molecular mechanism of insulin resistance in skeletal muscle after 2-AA treatment.

References- 1. Bassler BL, et al. Cell 125: 237-246, 2006. **2.** Cox CD, et al.J Clin Microbiol 9: 479-484, 1979.**3.** Roden M, et al. J Clin Invest 97: 2859-2865, 1996. **4.** Lee K, et.al. Metab Eng 2: 312-327, 2000.