A small volatile bacterial molecule triggers oxidative stress, apoptosis insulin resistance and concurs with mitochondrial dysfunction in murine skeletal muscle

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Introduction– A major cause of oxidative stress is reactive oxygen species (ROS) generation in cells, which is a by-product of energy production in mitochondria (1). Oxidative stress has been well recognized during infection (2) is hypothesized to contribute to the development of apoptosis. However, it is unknown whether compounds produced by bacteria during infection alter antioxidant defense thus producing oxidative stress leading to apoptosis in skeletal muscle. Also the role of mitochondrial oxidative stress in post-infection skeletal muscle dysfunction has not yet been studied. Our previous studies suggested that a volatile aromatic low molecular weight molecule, 2-amino acetophenone (2-AA), produced by the opportunistic human pathogen *Pseudomonas aeruginosa* promotes chronic infections possibly via oxidative stress due to increased ROS production. Here, we show that 2-AA affects the expression of genes involved in energy production metabolism and intermediate metabolism. We validated our results using an i*n vivo* and *ex vivo* nuclear magnetic resonance (NMR) spectroscopy method, which allows measurements of physiological and metabolic biomarkers in intact systems and has previously shown mitochondrial dysfunction in burns (3).

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 4 days following treatment with 2-AA.. All *in vivo* ^{31}P NMR experiments were performed in a horizontal bore magnet (proton frequency at 400 MHz, 21 cm diameter, Magnex Scientific, Varian, Palo Alto, CA, USA) using a Bruker Advance console. ^{31}P NMR spectra were analyzed using the MestReNova NMR software package (Mestrelab Research S.L., v. 6.2.1 NMR solutions, Website: www.mestrec.com). For the calculation of the ATP synthesis rate, information from the ^{31}P-NMR spectra and the previously mentioned biochemically measured concentration of ATP was used, as described by Forsen and Hoffman (4). Biopsies from the left gastrocnemius muscle (distal to the burn injury) were harvested at 6 h, 12 h, 1 d, 3 d, or 7 d post-burn (n = 3 for each time point) to examine changes in whole muscle gene expression. Animals were studied with HRMAS 1H NMR spectroscopy before and at 6 h, 24 h, and 72 h after burn trauma. The muscle tissue at the same site from unburned animals served as controls. 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.

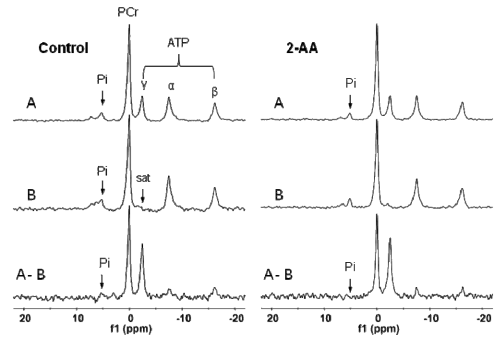

Figure1. NMR spectra of in vivo 31P NMR saturation-transfer performed on the hind limb skeletal muscle of mice.

Results– *In vivo* ^{31}P NMR spectroscopy shows that treatment with 2-AA does not significantly alters high-energy phosphates or the pH but reduces ATP synthesis. Although 2-AA did not alter high-energy phosphates or pH, our *in vivo* ^{31}P NMR results showed a significantly reduced rate of ATP synthesis and were complemented by genomic results showing downregulation of energy production. We used *in vivo* ^{31}P NMR to assess whether injection of 2-AA alters muscle concentrations of high-energy phosphates via perturbed mitochondrial functions. The levels of phosphomonoesters and inorganic phosphate and the ratio Pi/PCr are decreased in the 2-AA treated mice vs. the control, whereas the levels of PCr are higher in 2-AA mice vs. the control, but not significantly. The intramyocellular pH was not significantly different versus the control (7.26 ± 0.06 and 7.30 ± 0.05, respectively (P=0.60). ^{31}P NMR spectra were acquired from control and 2-AA treated mice at day 4, before and after saturation of the γATP resonance (Figure 1), This synthesis rate involves measurements from NMR and from a biochemical assay (ATP concentration measurement), and both were significantly decreased in the 2-AA treated mice. ATP synthesis rate of the PCr vs γ-ATP reaction was also significantly lower in 2-AA mice by 82% compared to control. Insulin resistance biomarker IMCLs as detected by NMR rise in gastrocnemius skeletal muscle of mice treated with 2-AA. Figure 2 shows representative ^{1}H-NMR spectra acquired from normal and 2-AA treated mice, and illustrates a notable rise in IMCLs upon treatment with 2-AA. Quantitative results of these measurements demonstrate a significant rise in ICML 4 days post- 2AA treatment. Also, the increased levels of bisallylic methylene fatty acyl protons (2.8 ppm) and vinyl protons (5.4 ppm), suggested that 2AA treatment results in apoptosis.

Discussion- ^{1}H NMR detects muscle lipid accumulation suggestive of apoptosis following 2-AA treatment. 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, $p < 0.05$). 2-AA downregulates the expression of energy production-related genes in skeletal muscle.Figure 3 shows the profiles of differentially expressed genes involved in energy production. Several components of the mitochondrial respiratory (proton transport and/or electron transport) chain were downregulated, including subunits of NADH dehydrogenases and ATP synthase. In conclusion 2AA affects skeletal muscle function. These findings implicate oxidative stress apoptosis and insulin resistance status associated with a mitochondrial dysfunction molecular signature in skeletal muscle following 2-AA treatment, which may be linked to 2-AA's ability to promote bacterial phenotypic changes associated with chronic inflammatory disease and infection.

References- 1. G. P. Bodey, et al. Rev. Infect. Dis., 1983, 5, 279–313. **2.** C. Koch and N. Hoiby, Lancet, 1993, 341, 1065–1069. **3.** KE,Padfield , et al Proc Natl Acad Sci U S A 102: 5368-5373, 2005. **4.** S. Forsen and R. Hoffman. Journal of Chemical Physics 39: 2892-2901, 1963.

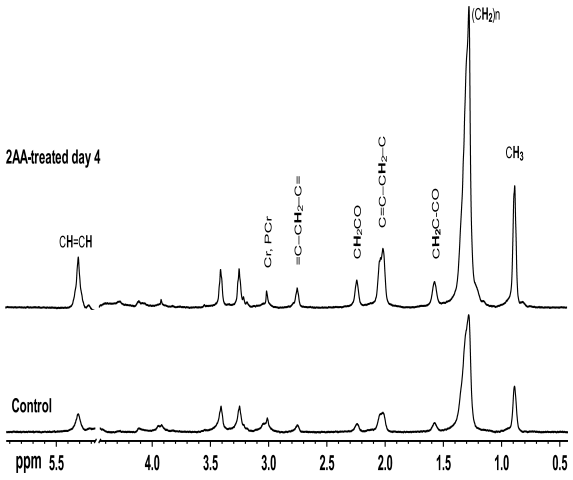

Figure 2. NMR spectra from ^{1}H-NMR HRMAS experiments performed on the gastrocnemius skeletal muscle specimens of mice. Resonance signals of lipids correspond to: terminal methyl CH_3 protons (0.9 ppm); acyl chain methylene protons $(CH_2)_n$ of intramyocellular lipids (IMCLs) (1.3 ppm); methylene protons CH_2C-CO (1.6 ppm); allylic methylene protons C=C-CH_2-C of monounsaturated fatty acyl moieties (MUFAs) (2.05 ppm); α methylene protons CH_2CO (2.25 ppm); diallylic methylene protons =C-CH_2-C= of polyunsaturated fatty acyl moieties (PUFAs), (2.74 ppm); N-methyl protons of phosphocreatine and creatine (3.0 ppm), respectively; and olefinic protons CH=CH (5.4 ppm) . The NMR spectra show increased biomarkers of insulin resistance IMCLs and apoptosis (PUFA and olefinic protons due to ceramide).

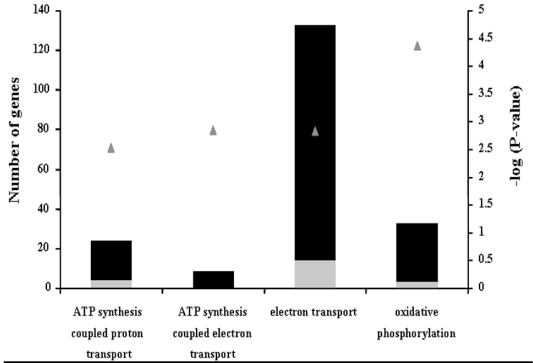

Figure 3. Differentially expressed genes involved in energy production. Grey boxes represent upregulation of the gene and black boxes represent downregulation of the gene compared with normal muscle. The expression of certain key genes is downregulated, consistent with the *in vivo* ^{31}P-NMR data