Nitrite Systems Biology: Transient Metabonomic Changes and Long-lasting Cardiac Proteomic, Redox, and Functional Alterations after a Single Spike in Nitrite

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Introduction: The nitrite anion (NO_2^-) is emerging as a critically important signaling molecule and modulator of cell function. Formed in the body through oxidation of the ubiquitous signaling molecule, nitric oxide (NO), or reduction of dietary nitrate, nitrite has been proposed to be involved in hypoxic vasodilation and has been demonstrated to confer protection against ischemic damage in several organ systems, including the heart. Yet, even now, almost nothing is known about how nitrite affects physiology or what are its molecular targets and effectors. Using an integrated, systems approach, including metabonomic, redox, proteomic and functional analyses, we have studied the time and dose-resolved impact on the heart of physiologically-relevant, brief elevations in systemic nitrite. We found that a single bolus application of nitrite induces a transient wave of elevated tissue nitrite levels and tissue nitros(yl)ation that elicits major, long-lasting alterations to the cardiac proteome and cardiac redox status that are associated with alterations in cardiac contractile recovery after global ischemia. We also have undertaken a global, proteomic characterization of the hearts of nitrite-treated animals by label-free quantitation and found distinct, large-scale, nitrite-dependent changes in protein expression.

Methods: **Methods**: Hearts were buffer-perfused and harvested from age-matched male Wistar rats at intervals from 0 min to 48 h following i.p. administration of three doses of nitrite. Concentrations of NO-related metabolites, ascorbate and glutathione redox status were determined by gas-phase chemiluminescence, HPLC and spectrophotometry. Contractile function of Langendorff-perfused hearts subjected to ischemia/reperfusion was assessed. Heart homogenates from the 24-h time point were subjected to both 2D-gel based and label-free LC/MS-based differential display. A preliminary report of the 2D gel experiments was presented earlier. Label-free quantitation by LC/MS was carried out by in-solution protein digestion, followed by replicate nano-flow LC/MS runs on an LTQ-Orbitrap™ MS (Thermo-Fisher Scientific) equipped with a Nanoacuity™ uPLC system (Waters) and a Nanomate™ robot (Advion). The LC-MS data was subjected to label-free quantitation using Progenesis™ LCMS software (Nonlinear Dynamics). Top scoring ions were manually inspected and validated for correct charge state and monoisotope assignment and peak area quantitation. Parallel LC-MS/MS was conducted and data were submitted to Mascot™ (Matrix Science) for query against the SwissProt and Trembl databases. Peptides from validated protein assignments were then matched to the top scoring ions of the same *m/z* from the Progenesis™ LCMS software. Protein IDs were functionally annotated with the GO-term annotation mining and graphical rendering software, Protein Function Junction (written in-house).

Results: We have examined the long-term effect on the heart of a brief treatment with nitrite, a little-understood ubiquitous physiological effector. In a rat model, designed to recapitulate the magnitudes and durations of normal physiologic bursts of nitrite production or consumption, we took a systems biology approach, combining proteomic analyses with metabonomic, redox and functional studies to forge a more complete understanding of nitrite biology. We found that a single bolus dose of nitrite induces a short spike in tissue nitrite levels and tissue nitros(yl)ation. Although this spike is transient, it elicits major, long-lasting alterations in cardiac redox status and cardiac contractile recovery after global ischemia. Using label-free quantitation by LC/MS, we extended our previous 2D-gel based proteomic analysis by finding that a transient spike in tissue nitrite levels has a significant, persistent and widespread impact on protein expression profiles. We observed that the vast majority of LC/MS features did not show significant changes. However, more than a thousand ion features, corresponding to hundreds of distinct proteins, *did* appear to undergo significant nitrite-dependent alteration in some way, suggesting that a single perturbation in physiologic nitrite homeostasis has a major impact on the cardiac proteome. In particular,

we report nitrite dose-dependent changes in the F1 ATPase and myoglobin proteins, both involved in energy metabolism and implicated in the past to potentially play a role in cardiac preconditioning and nitrite/nitric oxide metabolism.

Conclusions: We have found distinct, sizable, and significant nitrite-induced long-term changes in protein expression in the heart, changes that we believe provide evidence for major, fundamental and heretofore unappreciated changes in cardiac energy metabolism.

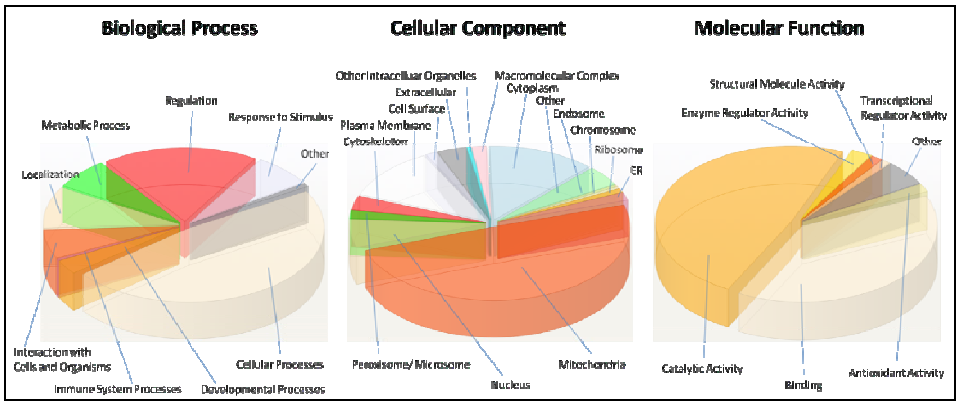

Fig. 1. Summary of gene ontology (GO) functional annotation of protein hits to the top 1000, hand-curated LC/MS ion features that showed significant changes across the sample set. Protein hits were obtained through inclusion-list directed LC-MS/MS, followed by rigorous protein assignments from the SwissProt database using Mascot. The GO-term annotations for each protein were mined and the contribution to each GO category was graphed. Individual pie charts show the GO super-categories.

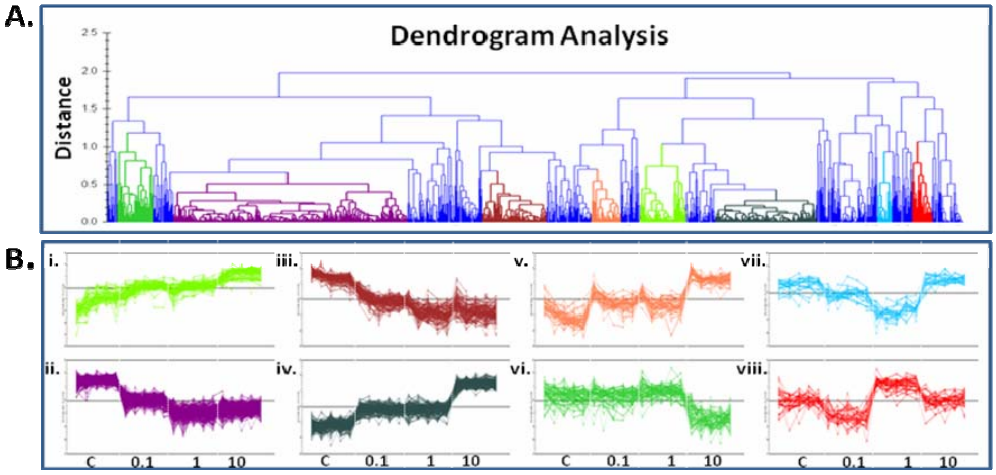

Fig. 2. Unsupervised clustering analysis of the entire set of assigned LC-MS ion features by similar expression level profiles. **A.** Dendrogram displaying relatedness of expression level profile of each ion feature. Highlighted in color are clusters of ions with the expression profiles shown in B. **B. i-viii,** Normalized relative expression level profiles of the feature clusters shown in A. across all the replicates of the samples: **C**, control (saline); **0.1**, 0.1 mg/kg nitrite; **1**, 1 mg/kg nitrite; **10**, 10 mg/kg nitrite.

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