Supplemental Results and discussion for:

Highly Multiplexed and Reproducible Ion Current-Based Strategy for Large-Scale Quantitative Proteomics and the Application to Protein Expression Dynamics Induced by Methylprednisolone in 60 Rats

Eslam Nouri-Nigjeh[1,2,#], Siddharth Sukumaran[1,#], Chengjian Tu[1,2], Jun Li[1,2], Xiaotao Duan[1,2], Debra C. DuBois[1,3], Richard R. Almon[1,2,3], William J. Jusko[1,2], Jun Qu[1,2,*]

[1] Department of Pharmaceutical Sciences, University at Buffalo, State University of New York, Buffalo, NY 14214

[2] New York State Center of Excellence in Bioinformatics and Life Sciences, Buffalo, NY 14203

[3] Department of Biological Sciences, University at Buffalo, State University of New York, Buffalo, NY 14260

# Authors contributed equally to this work

*Corresponding Author

Jun Qu, Ph.D.
Department of Pharmaceutical Sciences,
318 Kapoor Hall,
State University of New York at Buffalo,
Buffalo, NY 14214-1200.
Email:junqu@buffalo.edu

1. Gene Ontology annotations and temporal changes in biological processes.

We performed Gene Ontology analysis for all quantifiable proteins and for the 323 significantly-altered proteins (i.e. potential drug-responsive proteins). Of all quantifiable proteins with cellular component information available, 26% are associated with various membrane components, which is indicative of the excellent recovery of membrane proteins by our sample preparation method (**SI Figure S5**). Cellular locations of the drug-altered proteins are shown in **Figure 5A**. The percentages of the significantly altered proteins in relation to the total quantified proteins vary noticeably among different cellular components, suggesting that the drug actions may be location-specific. These results may reflect the known fact that CS regulate numerous signaling and metabolism pathways in specific cellular locations. These include highly-regulated protein degradation in lysosomes [1], very-long fatty acid chain metabolism in peroxisomes [2], long- and medium-size fatty acid chain metabolism in mitochondria [2], and protein synthesis machinery in the ribosome and endoplasmic reticulum [3]. **Figure 5B** shows the distributions of significantly-altered proteins by relevant biological processes. These altered proteins are consistent with reported CS hepatic drug actions [4].

The global measurement of the expression dynamics permits comprehensive investigation of the drug-altered biological processes with the dimension of time. To gauge the extent of alteration in each biological process at different time points, we investigated the profiles of several altered proteins in each category. Representative time-courses are shown in **Figures 5C-5F**. For all time-courses, little or no response was observed at very early time points (e.g. 0.5 and 1 h), followed by increases and then decays in the protein concentrations over the 66-h period post-dosing. MPL elicited sustained changes of proteins in "response to hormone stimulus" (**Figure 5C**), the majority of which belonging to the subcategories of “response to steroid”, and “corticosteroid

stimuli". Many temporal changes of hepatic metabolic processes, such as gluconeogenesis, occurred. The observed altered proteins in "gluconeogenesis" were all up-regulated, and the numbers peaked at 8 h (**Figure 5D)**. Most of the altered proteins in the "inflammatory/anti-inflammatory response" category were up-regulated and peaked at 12 h, as shown in **Figure 5E**. Further analysis revealed that most of these are among the acute response proteins, which are up-regulated by CS as part of their anti-inflammatory response [5]. The majority of proteins related to translation were up-regulated, as most sharply increased and peaked at 5.5 h followed by a decline (**Figure 5F**). This is consistent with the anabolic actions of CS in liver [6]. Interestingly, the temporal characteristics, such as the peak times and the rates of decline, are quite distinct among various biological processes, reflecting diverse regulatory mechanisms and dynamics.

2. Drug-responsive proteins in hepatic amino acid metabolism, gluconeogenesis and acute phase response

Previously, systematic investigations of drug responses were mostly conducted on enzyme activity and transcriptional levels but rarely on multiple protein levels due to the technical limitations. The method developed here provides a practical tool for comprehensive time series studies of protein-level changes underlying drug effects. Although the drug has a rapid clearance, e.g. the plasma concentration decreased to <1% of its maximum concentration at 5.5 h [7], our temporal proteomics data showed many protein expression changes occurring for up to 66 h, indicating that many biological cascades remained active well after the drug was cleared from the system. Investigation of the biological functions of the discovered drug-responsive proteins is our future plan; however, some key drug-responsive proteins involved in hepatic amino acid metabolism and gluconeogenesis are exemplified.

The CS-induced protein degradation, as illustrated in **Figure 6A**, provides the substrate for amino acid metabolism and gluconeogenesis[8]. Briefly, CS induce protein degradation through autophagocytosis via lysosomal machinery in hepatocytes [1], and the ATP-dependent ubiquitin-proteasome system in skeletal muscle [9], which provides amino acid substrates to liver for further amino acid metabolism and subsequent gluconeogenesis [10]. Three major aminotransferases that are CS-responsive were found in this study: alanine aminotranferase (AAT), cytosolic aspartate aminotransferase (cASAT), and tyrosine aminotransferase (TAT). The time-courses of the three aminotransferases as measured by the proteomic profiling are shown in **Figures 6B** and **6C**. Interestingly, although all three enzymes contain glucocorticoid response elements (GRE) in their conserved promoter regions and thus they can be up-regulated by CS-induced GR binding [11], the three enzymes peaked at different times with markedly different time profiles. While the induction of AAT and cASAT peaked near 30 h and continued until 48 h after drug dosing (**Figures 6B** and **6C**), the expression peaks for TAT are much narrower with maxima at much earlier times (5-8 h, **Figures 6C** and **6D**). These distinct temporal features reflect the complex biochemical and dynamic features of the regulation of these proteins in liver. One potential explanation in part for the differences in time-courses could be found in the differential turnover rates of these proteins. For example, cASAT has a much longer half-life (5-11 days) than TAT (~4 h) [12], which may account for the much wider response window of cASAT. Furthermore, in contrast to cASAT, the proteomic data showed no significant increase of mASAT, which is consistent with previous reports that mASAT is not responsive to CS [12] while CS can increase the expression of cASAT [12].

Hepatic gluconeogenesis is downstream of amino acid metabolism (**Figure 6A**). The recycling of carbons are controlled by three key enzymes: pyruvate carboxylase (PC),

phosphoenolpyruvatecarboxykinase (PEPCK), and fructose 2,6-bisphosphate (FBPase) [13] (illustrated in **Figure 6A**). Among these, PEPCK (also with a GRE region) is the rate-limiting enzyme [14]. The time courses of the three enzymes are shown in **Figure 6D.** Clearly, only the expression of PEPCK was elevated by MPL (peaking at 5.5 h), which is in agreement with previous observations of elevated mRNA [14] and enzyme activity [15] of PEPCK, while neither PC nor FBpase was altered. This result also correlates well with our observation that CS elevates glucose concentrations in plasma [16].

Among the significantly up-regulated proteins in inflammatory responses, 7 proteins are associated with acute phase response including serpin A3n, serpin A1, alpha-2-macroglobulin, argininosuccinate synthase, complement C4, Haptoglobin, and signal transducer and transcription activator 3 (STAT3). The increase of these proteins may play a significant role in tissue and organ protection in response to diverse stimuli [17]. Serpins and alpha-2-macroglobulin are antiproteases involved in fibrinolysis, the inhibition of blood clots, and may increase in circulation in response to various stimulations and inflammatory stresses [18]. Complement C4 is a part of the complement system and is critical in host defense [19]. Haptoglobin is a key transport protein in hepatic recycling of heme iron and tissue protection [20]. Argininosuccinate synthase is an acute phase response protein involved in the control of blood pressure [21]. STAT3 showed a time profile with significant up-regulation, showing its peak expression between 8-18 h. STAT3 is of interest because it is a transcription factor responsible for regulation of many acute phase response proteins [22]. The mRNA expression of STAT3 was also found to be up-regulated by MPL in previous work [23]**.** The concordant up-regulation of STAT3 at both mRNA and protein levels supports the receptor/gene-mediated mechanism for enhanced expression of STAT3 at the transcriptional level, which in turn may contribute to the elevation of the downstream acute phase

response proteins observed here. The up-regulations of these proteins were supported by the reduction of white blood cells after MPL dosing [24].

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