Untargeted Plasma Metabolite Profiling Reveals the Broad Systemic Consequences of Xanthine Oxidoreductase Inactivation in Mice

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Abstract

A major challenge in systems biology is integration of molecular findings for individual enzyme activities into a cohesive high-level understanding of cellular metabolism and physiology/pathophysiology. However, meaningful prediction for how a perturbed enzyme activity will globally impact metabolism in a cell, tissue or intact organisms is precluded by multiple unknowns, including in vivo enzymatic rates, subcellular distribution and pathway interactions. To address this challenge, metabolomics offers the potential to simultaneously survey changes in thousands of structurally diverse metabolites within complex biological matrices. The present study assessed the capability of untargeted plasma metabolite profiling to discover systemic changes arising from inactivation of xanthine oxidoreductase (XOR), an enzyme that catalyzes the final steps in purine degradation. Using LC-MS coupled with a multivariate statistical data analysis platform, we confidently surveyed >3,700 plasma metabolites (50–1,000 Da) for differential expression in XOR wildtype vs. mice with inactivated XOR, arising from gene deletion or pharmacological inhibition. Results confirmed the predicted derangements in purine metabolism, but also revealed unanticipated perturbations in metabolism of pyrimidines, nicotinamides, tryptophan, phospholipids, Krebs and urea cycles, and revealed kidney dysfunction biomarkers. Histochemical studies confirmed and characterized kidney failure in xor-nullizygous mice. These findings provide new insight into XOR functions and demonstrate the power of untargeted metabolite profiling for systemic discovery of direct and indirect consequences of gene mutations and drug treatments.

Introduction

Metabolomics, the systems biology of small molecules, has emerged in the post-genomic era as a powerful tool for profiling differences in the expression of structurally diverse molecules in complex biological mixtures [1–6]. In comparison to other “omics” technologies, metabolomics offers the most proximal approach for defining key differences between cell phenotypes. A significant challenge to metabolomics is the untargeted discovery of changes in metabolite expression that are hidden in large data matrices, acquired using high-throughput analytical procedures. Notwithstanding, untargeted profiling offers the potential to discover disease-associated and drug-induced changes in the expression of diverse small molecules in a biological fluid or tissue, revealing biomarkers that can be used to inform on disease progression or the efficacy of clinical treatments. In this regard, metabolomics can serve as an extension of traditional (targeted) clinical analyses, providing a far more comprehensive snapshot of the functional status of a complex biological system [7]. The resulting identification and quantification of disease and treatment-associated metabolites can trigger unanticipated hypotheses and novel mechanistic insights.

The ideal metabolomic platform would be able to accurately quantify the broad structural spectrum of small molecules that reside in cells, tissues and biofluids, at their biological levels – an elusive challenge. Notably, the extreme complexity of most biological sample matrices demands multiple metabolite separation modes for broad analytical detection and molecular identification to be effectively approached. To meet this challenge, recent developments in high-performance liquid chromatography (LC) separations have provided promising platforms for the rapid resolution of thousands of metabolites in biological extracts [8–13]. Combined with advances in MS instrumentation that enable rapid acquisition of high-resolution accurate mass spectra and chemometric platforms for automated data analysis and interpretation, effective metabolomic strategies for profiling and identifying diverse metabolites have emerged [14–17].

Xanthine oxidoreductase (XOR) is a molybdoenzyme containing enzyme that catalyzes the two terminal steps in purine
catabolism, oxidizing hypoxanthine initially to xanthine and finally to uric acid. XOR exists in two interconvertible forms that each yield ureate: xanthine oxidase (XO) and xanthine dehydrogenase. The distinction is that XO uses molecular oxygen as its electron donor and generates superoxide as a product, whereas xanthine dehydrogenase preferentially reduces NAD+ without superoxide generation. Xanthine dehydrogenase is considered to be the predominant physiological form of XOR in cells. Over the past two decades, there has been growing interest in the pathophysiological roles of XOR in a wide range of disease processes [18–24]. For example, upregulation of XOR activity has been implicated in various ischemic diseases and leads to hyperuricemia, among other manifestations of reperfused tissue injury [25]. Genetic deficiency of xor in mice has been linked to renal fibrosis and disease [26,27].

In this investigation, we sought to inventory the metabolic consequences that occur in plasma metabolite levels in mice with a functional deficiency in XOR and evaluate the capability of an untargeted LC-MS-based platform for profiling both predicted and unobserved changes. Although the direct enzymatic role of XOR in mammalian purine degradation is established from studies of the isolated enzyme [28–32], the inferred purine-independent actions of XOR [26,29,33,34] and secondary downstream metabolic perturbations that can arise from purine-dependent and -independent actions remain to be defined.

In this post-reductionist era of protein science, there is a growing appreciation for the idea that protein functions should be studied and understood in the context of native physiological microenvironments [35]. Since plasma provides the predominant reservoir of metabolites for cellular ingress and egress, and metabolites are the final downstream effectors of genes, the plasma metabolome can be used to globally inform on the organismal consequences of altered gene expression and drug treatments. Thus, global plasma metabolite profiling offers the potential to provide a broad overview of effects that may be induced by drug actions and altered gene expression, rendering plasma metabolite profiling as a promising technology for in vivo functional genomics/proteomics. We demonstrate in this report that untargeted plasma metabolite profiling, using LC-MS with advanced chemometric data mining, reveals predicted alterations in purine degradation, but also identifies unanticipated functions of XOR and discovers plasma biomarkers that inform on emergent kidney failure.

Results

Technical Assessment of Plasma Metabolite Profiling Performance

Plasma, the reservoir of circulating metabolites, can provide a systems level read-out of the physiological state of an organism. To determine the technical reproducibility of untargeted analyses of plasma, we performed 56 repeat analyses on a 0.2 µl injected volume of a single human plasma (5 µl total injection volume, from a 25-fold diluted plasma sample) and analyzed the consistency of findings. Using data acquired by ANP chromatography with positive ion detection, an overlay of total ion chromatograms from the 56 repeat analyses revealed <10% run-to-run deviation (Fig. 1A). From these analyses, we tracked variations in the detection levels of 374 distinct metabolites that span the mass and chromatographic space of the dataset and additionally differ in ion abundance over 3-orders of magnitude (Fig. 1B). Considering the 56 repeated measurements of all 374 metabolites, normalized ion counts showed a mean coefficient of variation (CV) of 6.51%, median CV of 5.91% and high/low CV of 0.72% and 15.01%, respectively, considering all features. In accord with this relatively low overall CV for metabolite quantification, Fig. 1C shows extracted ion chromatography depicting the reproducibility in quantifying some typical plasma metabolites overlaying the 56 repeat measurements of cognate peaks. The high technical reproducibility of plasma metabolite quantification using this platform would predictably allow for confident identification of biologically-relevant changes that are ≥20% of control levels.

Metabolic Pathway Perturbation Elicited by xor Gene Deletion

Employing a multimode analytical platform for expanded feature coverage relative to that shown in Fig. 1, we analyzed plasma from xor WT and xor KO mice in both positive (POS) and negative (NEG) ionization MS detection modes, using both hydrophilic (ANP, aqueous normal phase) and hydrophobic (RP, reversed-phase) chromatographic separations. As shown in Fig. 2, each of the four data acquisition modes contributed substantially to feature coverage and only modest overlap was observed between detection modes. Among the four detection modes, ANP-POS LC/MS provided quantification of the greatest number of features (2,004), followed by RP-NEG (1,123), RP/POS (611) and ANP-NEG (406). Combining data from the 4 detection modes, a total of 3,716 unique features were quantified with 100% frequency in either xor WT, KO, or both. Table 1 lists the verified identities of 52 metabolites found to be differentially-expressed in KO vs. WT plasma, with p<0.05 and fold-change >2.0. As indicated in Table 1, xor gene deletion was associated with a significant accumulation of some plasma features (denoted by a positive Log2 value) and a decrement in others (denoted by a negative Log2 value). In accord with expectations, untargeted plasma metabolite profiling revealed that xor gene deletion is associated with marked increases in circulating levels of xanthine, hypoxanthine, xanthosine and inosine, (XOR substrates and upstream metabolites in the purine salvage pathway), and depletion of uric acid and allantoin (the XOR product and downstream metabolite). Unexpectedly, xor gene deletion was also consistently associated with various other metabolic pathways, seemingly unrelated to purine metabolism; these include intermediates in metabolic pathways for pyrimidines, the tricarboxylic acid (Krebs) cycle, urea cycle, tryptophan, nicotineamida and phospholipids. Additionally, a series of small molecule biomarkers of kidney dysfunction [36] were observed to accumulate in plasma from xor KO mice, including creatinine, phenol, phenylsulfate, indoxyl, indoxyl sulfate, quinol, quinol sulfate, N-methyl-1-pyridone-5-carboxamide, N-Methyl-1-pyridone-2-carboxamide and hippuric acid. The observed array of predicted metabolic changes upstream and downstream of XOR enzyme activity confirmed the potential of metabolite profiling as a systems biology approach to identify gene functions, whereas unanticipated findings suggested the possibility of unappreciated XOR activities and unrecognized interactions of the perturbed purine salvage pathway with other metabolic pathways.

Genotype-specific Metabolite Profiles Across Multi-treatment Groups

Further studies were performed to assess the impact of xor gene dosage on the plasma metabolome. Toward this end, we compared plasma metabolite profiles from xor WT and KO mice with plasma from xor heterozygous mice (HET). Further, we sought to determine the extent to which the observed effects of xor...
gene-deletion on plasma metabolites would be recapitulated in xor WT mice after pharmacological inhibition of XOR activity, using the clinically-used drug, allopurinol (allopurinol treated group designated as WTA). Considering only ANP-POS metabolite profiling data in this expanded investigation, we observed a total of 8,360 unique aligned features in 18 plasma samples included in the study. Of the 8,360 observed features, 1,240 were quantified with 100% frequency in at least one group. Fig. 3 depicts the changing patterns of expression of these 1,240 reproducibly identified plasma features across groups, comparing measurements from WT, HET, KO and WTA mice. These profiles are plotted in Fig. 3 as the average abundance in each group. Results indicate striking similarities in the metabolite profile patterns of XOR-inactivated mice (whether allopurinol-treated or xor-null) and distinct differences from XOR-expressing mice (whether WT or HET). Among the 1,240 metabolites, relative levels of xanthine, hypoxanthine, uric acid and allantoin displayed pattern changes predicted for XOR protein expression patterns in xor WT, HET, KO and WTA. Numerous additional metabolites were observed to follow identical plasma expression patterns as that of xanthine, hypoxanthine, uric acid and allantoin, suggesting unappreciated metabolic linkages.

Because untargeted metabolomic studies are exploratory in nature and often result in large data sets, data analysis can benefit markedly from interpretation using multiple statistical and visualization tools. The goal of unsupervised pattern recognition is to identify and display natural groupings in the data without imposing any preconception about class membership. The PCA score plot, shown in Fig. 4A, provides a three-dimensional visualization of similarities and differences for all 1,240 plasma metabolites recognized in 100% of samples from at least one murine group. Each principal component (PC) represents the weighted linear combination of original LC/MS data, shown in the loadings plot (Fig. 4B). If two groups are found to differ in metabolite expression along the PC1 axis, then the loadings plot for PC1 can be used to determine which features contribute to the greatest extent in producing this difference. PCA findings show that xor WT and KO groups are clearly distinguished, reflecting distinct xor genotype-based differences in the respective plasma metabolomes. The xor HETs exhibited mixed pattern changes in their metabolite profiles, with 3/6 more closely resembling WT
and the remaining 3/6 resembling KO. In contrast, xor WTA and KO are essentially inseparable along PC1, but clearly distinguished along PC2, reflecting a predominant component of XOR phenotype (activity) similarity in this parameter, despite genotype dissimilarity. This suggests that the genetic background of WTA may be inferred from PC2, while the influence of XOR inactivity may be revealed by PC1. Indeed, the dual contribution of XOR activity (PC1) and xor genotype (PC2) may be explicitly projected and visualized by the PCA score plot. It can also be seen in Fig. 4A that there is a clear trajectory from WT to HET, then KO, consistent with progressive changes in metabolism with increasing activity (PC1) and may be revealed by PC1. Indeed, the dual contribution of XOR activity (i.e., contribute most profoundly to observed metabolic differences (i.e., S-adenosylmethionine, N-methylnicotinamide, and 1-methylhistidine). Additionally, KO and WTA mice exhibited a series of differentially expressed metabolites showing opposite changes, compared to WT, suggesting that these differences do not arise as a simple consequence of XOR enzyme inactivity (Fig. 5A). These discordant features included glycerophosphocholines, ceramides and some of the urea cycle and polyamine pathway metabolites, which were significantly elevated in KO mouse plasma, but unchanged after allopurinol treatment (relative to WT).

Physiological Correlations

Untargeted plasma metabolite profiling revealed biomarkers consistent with kidney failure. To investigate the veracity of this possibility, we compared renal function and histology in xor KO mice vs. xor HET and WT mice. Using standard assays for renal function, xor KO mice exhibited conspicuous kidney failure at 14 days of life, when plasma was acquired for metabolite profiling. Kidney failure was evidenced by a marked elevation of plasma...
Table 1. Differentially-expressed plasma metabolites in xor KO vs. WT mice.

| Metabolite/Pathway              | Molecular Formula | Mass (measured) | Mass deviation (ppm) | RT (min) | Fold-change (Log2)* | p-value | Major detection mode |
|---------------------------------|-------------------|-----------------|----------------------|----------|---------------------|---------|----------------------|
| **Purine pathway**              |                    |                 |                      |          |                     |         |                      |
| Xanthine                        | C5H4N4O2           | 152.0337        | −2.7                 | 1.621    | 16                  | 6.1e−5  | ANPPOS               |
| Xanthosine                      | C10H12N4O6         | 284.0771        | −4.8                 | 1.759    | 16                  | 6.1e−5  | ANPPOS               |
| Hypoxanthine                    | C5H4N4O            | 136.0389        | −3.1                 | 2.022    | 8.08               | 0.010   | ANPPOS               |
| Adenosine                       | C10H13N5O4         | 267.0971        | 0.0                  | 2.476    | 3.09               | 0.021   | ANPPOS               |
| Inosine                         | C10H12N4O5         | 268.0813        | −0.8                 | 1.756    | 16                  | 0.025   | RNNEG                |
| Uric acid                       | C5H4N4O3           | 168.0287        | −2.3                 | 1.678    | −16                 | 6.2e−4  | ANPPOS               |
| Allantoin                       | C4H6N4O3           | 158.0437        | −0.1                 | 1.574    | −16                 | 3.2e−4  | ANPPOS               |
| **Pyrimidine pathway**          |                    |                 |                      |          |                     |         |                      |
| Uridine                         | C9H12N2O6          | 244.0694        | −0.2                 | 1.103    | 16                  | 2.0e−5  | RPNEG                |
| Dihydouridine                   | C9H14N2O6          | 246.0846        | −2.6                 | 1.695    | 2.27               | 0.0011  | ANPPOS               |
| Dihydouracil                    | C4H6N2O2           | 114.0433        | 3.9                  | 1.533    | 2.13               | 0.0042  | ANPPOS               |
| **Citric acid cycle**           |                    |                 |                      |          |                     |         |                      |
| Malate                          | C4H6O5             | 134.0215        | 2.7                  | 1.018    | 16                  | 5.7e−4  | RP-NEG               |
| Aconitate                       | C6H6O6             | 174.0170        | 3.5                  | 1.191    | 3.69               | 3.0e−4  | RP-NEG               |
| Fumarate                        | C4H4O4             | 116.0110        | −0.5                 | 1.050    | 3.72               | 0.022   | RPNEG                |
| Citrate                         | C6H8O7             | 192.0270        | 0.2                  | 1.155    | 2.31               | 0.0050  | RP-NEG               |
| Ketoglutarate                   | C5H6O5             | 145.038         | −3.5                 | 1.057    | 5.64               | 8.2e−4  | RP-NEG               |
| Pyruvate                        | C3H4O3             | 88.0160         | −1.2                 | 1.144    | 1.94               | 0.0058  | RP-NEG               |
| **Tryptophan pathway**          |                    |                 |                      |          |                     |         |                      |
| Kynurenic acid                  | C10H7NO3           | 189.0426        | −1.3                 | 1.695    | 3.82               | 0.0039  | ANPPOS               |
| Xanthurenic acid                | C10H7NO4           | 205.0374        | −0.1                 | 3.288    | 3.35               | 0.042   | ANPPOS               |
| Indoxyl                         | C8H7NO             | 133.0529        | −1.4                 | 3.534    | 2.37               | 0.0086  | ANPPOS               |
| Indoxylsulfate                  | C8H7NO45           | 213.0096        | −1.7                 | 3.536    | 2.19               | 0.0078  | RPNEG                |
| Tryptophan                      | C11H12N2O2         | 204.0892        | 2.2                  | 4.420    | −2.27              | 0.041   | ANPPOS               |
| **Urea cycle and related**      |                    |                 |                      |          |                     |         |                      |
| Argininosuccinate               | C10H18N4O6         | 290.1250        | 0.7                  | 11.363   | 16                 | 4.2e−5  | ANPPOS               |
| Fumarate                        | C4H4O4             | 116.0110        | −1.5                 | 0.755    | 3.72               | 0.022   | RPNEG                |
| Proline                         | C5H9NO2            | 115.0636        | −3.7                 | 6.996    | 1.29               | 3.4e−4  | ANPPOS               |
| Ornithine                       | C5H12N2O2          | 132.0899        | −0.3                 | 12.534   | 1.25               | 2.7e−4  | ANPPOS               |
| N-Acetyliminorhithine           | C7H14N2O3          | 174.0994        | −3.1                 | 8.599    | 1.59               | 0.045   | ANPPOS               |
| Citrulline                      | C6H13N3O3          | 175.0961        | −2.3                 | 8.601    | 1.22               | 0.031   | ANPPOS               |
| Homocitrulline                  | C7H15N3O3          | 189.1124        | −3.9                 | 8.576    | 1.72               | 5.7e−5  | ANPPOS               |
| Urea                            | C4H2NO2            | 60.0329         | −8.0                 | 1.873    | 1.74               | 3.8e−5  | ANPPOS               |
| **Nicotinamide pathway**        |                    |                 |                      |          |                     |         |                      |
| N-Methylnicotinamide            | C7H8N2O            | 136.0635        | 0.0                  | 11.295   | 2.24               | 0.033   | ANPPOS               |
| N-Methyl-2-pyridone-5-carboxamide| C7H8N2O2          | 152.0583        | 0.2                  | 2.450    | 1.74               | 6.2e−5  | ANPPOS               |
| N-Methyl-4-pyridone-5-carboxamide| C7H8N2O2         | 152.0583        | 4.7                  | 1.867    | 2.2                | 1.8e−4  | ANPPOS               |
| Nicaminide                      | C6H6N2O            | 122.0483        | −2.7                 | 1.846    | −1.55              | 2.2e−5  | ANPPOS               |
| **Phospholipid metabolism**     |                    |                 |                      |          |                     |         |                      |
| Pantotenic Acid                 | C9H17NO5           | 219.1111        | −1.8                 | 1.357    | 2.63               | 1.6e−4  | ANPPOS               |
| Myristoyl-lysophosphatidylcholine| C22H46N7O7P       | 467.3011        | 0.5                  | 7.696    | 2.21               | 2.0e−4  | ANPPOS               |
| Dioleoyl-phosphatidylethanolamine| C41H78NO18P     | 743.5447        | 2.4                  | 1.748    | 2.14               | 1.0e−4  | ANPPOS               |
| Dioleoyl-phosphatidylcholine     | C41H84NO18P        | 785.5937        | −0.4                 | 4.560    | 1.65               | 0.021   | ANPPOS               |
| Glycerolphosphocholine           | C8H20NO6P          | 257.1035        | 2.8                  | 12.172   | −1.88              | 0.0080  | ANPPOS               |
| **Renal disease markers**       |                    |                 |                      |          |                     |         |                      |
| Creatinine                      | C4H7N3O            | 113.0590        | 2.5                  | 6.776    | 16                 | 1.0e−7  | ANPPOS               |
| Quinol                           | C6H6O2             | 110.0362        | 0.6                  | 2.416    | 3.78               | 0.0039  | RPNEG                |
Table 1. Cont.

| Metabolite/Pathway         | Molecular Formula | Mass (measured) | Mass deviation (ppm) | RT (min) | Fold-change (Log2)* | p-value | Major detection mode |
|---------------------------|-------------------|-----------------|----------------------|----------|---------------------|---------|----------------------|
| Quinol sulfate            | C6H6O5S           | 189.9929        | 1.0                  | 2.417    | 2.49                | 0.0059  | RPNEG                |
| Hydroxyhydroquinone        | C6H6O3            | 126.0314        | 2.7                  | 1.670    | 1.08                | 0.0021  | ANPPOS               |
| Phenol                     | C6H6O             | 94.0420         | –3.6                 | 2.902    | 2.64                | 0.039   | RPNEG                |
| Phenylsulfate              | C6H6O4S           | 173.9985        | –1.4                 | 2.901    | 2.29                | 0.046   | RPNEG                |
| Hippuric acid              | C9N9N03           | 179.0609        | –1.9                 | 1.131    | 2.55                | 0.0066  | ANPNEG               |
| 2-hydroxyphenylacetate     | C8H8O3            | 152.0472        | 0.1                  | 7.986    | 1.21                | 0.048   | RPNEG                |

*Positive values indicate increased plasma levels in xor KO vs. WT, whereas negative values denote a decreased plasma levels in xor KO vs. WT.

1Identified as mixtures of 2-hydroxyadipic acid and 3-hydroxyadipic acid.

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creatinine (1.22±0.15 mg/dL vs. 0.25±0.03 mg/dL in WT; Fig. 6A) concomitant with severe hypouricemia (Fig. 6B). Additionally, kidney size was markedly diminished at 14 days, compared to xor WT and HET littermates (Fig. 6C). The build-up in plasma xanthine and hypoxanthine that we observed by untargeted metabolite profiling in xor KO mice was associated with the deposition of birefringent crystalloid concretions in the renal tubules, focally present as sludge in the renal pelvis (Fig. 6D–F). These crystalloids were occasionally observed to penetrate tubular epithelial cells, but were mostly intraluminal and partially-to-completely obliterative (i.e., resulting in variable degree of tubular dilation, predominantly affecting the outer cortex or the papillary tips). There was also a variable degree of papillary attenuation, suggestive of obstructive uropathy. In contrast, wildtype mice showed no significant kidney pathology and heterozygote mice exhibited mild focal simplification of glomerular capillary tufts with much more modest cortical tubulointerstitial scarring. Taken together, these analyses verified aberrant kidney structure and function in xor gene-deleted mice, in accord with predictions based on observed metabolic biomarkers of kidney dysfunction inferred from untargeted plasma profiling analysis.

Discussion

XOR is not only the terminal enzyme in purine metabolism, catalyzing the oxidation of purine metabolites to uric acid, but it has also been implicated as a determinant of adipogenesis and peroxisome proliferator-activated receptor-γ activity [33], cyclooxygenase-2 gene expression [26], catalytic conversion of nitrate and nitrite to nitric oxide [29,34], catalytic hydroxylation of a wide range of N-heterocyclic and aldehyde substrates [29], and may also provide an endogenous source of ROS formation for cell signaling [37,38]. XOR activity is subject to both pre-transcriptional and post-transcriptional control by mechanisms that are modulated by hormones, cytokines and oxygen tension [29]. Given the multifunctional and poorly-defined systemic roles of XOR, we employed global plasma metabolite profiling in attempt to define XOR functions in mice and additionally, to assess the efficacy of untargeted profiling as an effective discovery approach. Taken together, our findings revealed unexpectedly broad XOR.

Figure 3. Patterns of change in murine plasma metabolite levels associated with XOR activity suppression. Striking similarities in plasma metabolite profiles are depicted in mice with deficient XOR activity (arising from either homozygous xor gene deletion or from allopurinol treatment; KO and WTA, respectively) vs. XOR-expressing mice (both xor HET and WT). Each line represents the levels of one of 1240 metabolites (quantified in all members of at least one group by ANP-POS), connected by its normalized abundance across groups. Color hues represent a heat map of normalized abundances (0.0–5.0), ranging from blue (cold) to red (hot).

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activity-dependent alterations in the plasma metabolome, uncovering primary, secondary, and tertiary biochemical derangements that apparently arise from deficient XOR enzyme activity. Further, plasma metabolome derangements in xor-deleted mice indicated end-stage kidney failure, which was confirmed by histochemically-defined renal pathologies and marked hypercrea-
tinemia and hypouricemia. Notably, xor gene KO was associated with interstitial fibrosis in the kidney and renal tubular accretions containing xanthine/hypoxanthine crystalloid matter (Fig. 6), consistent with a prior report [27].

A schematic model is presented in Fig. 7 that seeks to link primary, secondary and tertiary metabolic events that arise from xor gene deletion, as molecular underpinnings for renal pathophysiological consequences. In addition, a curated metabolic network that quantifies XOR gene-deletion induced changes is shown in supplemental Fig. S2. Primary metabolites that are upstream and downstream to XOR in the purine salvage pathway (i.e., xanthine, hypoxanthine and uric acid) were consistently identified in our study by untargeted profiling as among the most influential features that distinguish the plasma metabolome of both xor KO and allopurinol-treated mice from WT mice. We posit that the accumulation of primary XOR substrates (i.e., xanthine and hypoxanthine), drives an accelerated flux through the purine salvage pathway, accounting for substantial observed increases in secondary purine metabolite levels in plasma (i.e., xanthosine, inosine, adenosine and derived species). Accumulated xanthine and hypoxanthine provide abundant substrates for purine nucleoside phosphorylase 1 (PNP1), a purine salvage pathway enzyme that catalyzes the reversible phosphorolysis of purine nucleosides and inorganic phosphate, yielding the corresponding purine bases and ribose-1-phosphate. Notably, in addition to its service in the purine salvage pathway, PNP1 also catalyzes the inter-conversion of deoxyuridine to uracil (pyrimidine pathway) and N-ribosyl-nicotinamide to nicotinamide (nicotinamide metabolism). Given this involvement of PNP1 in multiple purine-independent pathways, elevated levels of secondary purine metabolites would predictably compete for the occupancy of PNP1, thereby interfering with the metabolism of other biomolecules that similarly rely on PNP1—this includes aberrant levels of pyrimidine and nicotinamide intermediates, considered in Fig. 7.
to be a tertiary metabolic consequence of XOR insufficiency. Notably, as shown in Table 1, pyrimidine and nicotinamide pathway metabolites were significantly perturbed in xor KO mice and this finding extended to allopurinol-treated xor WT mice. Competition for PNP1 by accumulated purine intermediates may also explain a prior report demonstrating significantly altered levels of uridine, uracil, cytosine, and nicotinamide in xor-deleted Drosophila [39].

The molecular basis for renal failure in mice with suppressed XOR activity is obscure, but is presumed to be triggered by primary, secondary, or tertiary metabolic perturbations that result from XOR deficiency, perhaps further enhanced in xor KO mice by a defect in kidney development. Notably, beyond the clinical gold standard biomarkers of kidney failure, creatinine and urea, untargeted plasma metabolite profiling identified numerous additional upregulated levels of biomarkers that are associated with kidney failure – these include phenols, indoxyl and its phase-II metabolite indoxylsulfate, quinol and its phase-II metabolites quinol sulfate [40] and quinol glucononide, hippuric acid, hydroxyphenylacetate, trimethylamine-N-oxide. Polyamines, found at elevated level in renal disease patients' plasma [41], were also significantly elevated in xor KO mice. Indoxyl sulfate is a protein-bound uremic toxin that is mainly produced by gut bacteria-mediated decomposition of dietary tryptophan in the intestine and accumulates in the plasma of patients with chronic kidney disease [40]. Serum indoxyl sulfate was shown to be associated with mortality in chronic kidney disease patients [42]. Impaired clearance of uremic toxins by the kidney results in their...
accumulation in plasma, with the potential to trigger systemic oxidative stress. Accumulation of these uremic toxins in plasma of xor KO mice may be either a cause or effect of renal kidney failure.

Less well explained metabolite changes associated with XOR inactivation in our study included increases in the apparent activity of the oxidative tryptophan/kynurenine pathway, TCA cycle and urea cycle (Table 1). Prior literature suggests that these metabolic perturbations could arise from kidney failure, possibly triggered by primary, secondary or tertiary metabolic consequences of insufficient XOR activity. For example, it has been shown that kynurenic acid, xanthurenic acid and kynurenine 3-hydroxylase activity are significantly increased in chronic renal failure in patients [43–45]. Moreover, the accumulation of L-kynurenine and its degradation product was found to be proportional to the severity of renal failure and correlated with the plasma concentration of renal insufficiency biomarker, creatinine [43]. Similarly, plasma levels of urea and the urea cycle intermediates ornithine and citrulline are elevated in renal failure patients, while arginine and aspartate have been shown to remain relatively constant [46]. We found that some urea cycle intermediates are also elevated in xor KO mouse plasma (ornithine, citrulline and argininosuccinate), along with some downstream metabolites (polyamines and proline), while levels of arginine and aspartate did not change (Table 1). Thus, kidney failure in xor KO mice may underlie our observed plasma accumulation of tryptophan oxidation products and urea cycle metabolites and related species. Finally, serum levels of the TCA cycle intermediates citrate, fumarate, oxaloacetate and malate have been reported to be significantly increased in human renal disease patients and appear to be correlated with disease progression and severity [47]. Thus, the elevated level of TCA cycle intermediates we observe in xor KO mice is in accord with that seen in human renal disease patients and may also be a consequence of kidney dysfunction, contributing to the failure of xor KO mice to thrive.

Plasma phospholipids, especially phosphatidylycerolines and ceramides were observed to increase in xor KO mice, but not in allopurinol treated WT mice (Fig. S1). Increased plasma phosphatidylycerolines in KO mice is in accord with a prior report demonstrating an essential developmental role for xor gene expression during adipogenesis in mouse embryos [33]. Enhanced expression of adipogenesis-related genes was previously described in xor-disrupted mice with the accumulation of glyceride-rich lipids in the renal tubules [27]. In contrast, allopurinol treatment was not found to inhibit adipogenesis or lipid-droplet formation in a human XOR transfected pre-adipocyte cell line [33], raising the possibility that the adipogenic activity of xor may be limited to a discrete period during embryonic development. Alternatively, the adipogenic activity of xor may be mediated by the XDH activity of XOR, which is not inhibited by allopurinol. Notably, mammalian XOR exists in two interconvertible forms, XO and XDH, with the

Figure 7. Schematic model for XOR insufficiency-induced primary, secondary and tertiary metabolic consequences, culminating in kidney failure. The present study found metabolites in bold to be upregulated in xor KO mice and non-bold metabolites downregulated. This model provides a framework that seeks to reconcile observed systemic consequences of xor gene KO on the murine plasma metabolome – see Discussion for details.

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latter being the predominant form in vivo. Allopurinol, and its active metabolite oxypurinol, inhibits the XO activity by binding to the molybdenum site of the enzyme [49], but not the FAD site of XDH which has a higher NADH oxidizing activity. Given that XDH activity remains unperturbed with allopurinol treatment, increased phospholipids in xor KO suggests the possible mediation by the NADH oxidizing activity of XDH for adipogenesis and PPAR-α activity.

The broad metabolic consequences we observed with high-dose acute allopurinol treatment in mice warrants consideration for allopurinol use in patients. Initially limited to the treatment of patients with gout, allopurinol therapy has since been extended to chronic stable angina [49], stroke [50], congestive heart failure [51] and even chronic kidney disease [52]. Animal studies have also demonstrated efficacy of allopurinol therapy in type II diabetic mice [53] and in kidney diseases with a significant tubulointerstitial component [54], among others. Escalating doses of allopurinol have been advocated by some investigators, even in the face of recognized renal insufficiency [49,55,56]. Although allopurinol intolerance is usually mild, it occurs in 10–15% of patients [57] and only in rare cases has more severe complications been reported. Among those more severe complications are Stevens-Johnson and hypersensitivity syndromes [57]. DRESS (drug reaction and eosinophilia syndrome) [58] and an increase in the number of opportunistic infections in patients with inflammatory bowel disease who receive allopurinol in combination with thiopurine therapy [59]. Admittedly, severe adverse effects of allopurinol in patients are rare; however subclinical renal consequences may be more widespread. The data presented herein provide a compendium of plasma biomarkers that may be used to identify subclinical toxicity of allopurinol and also a cautionary note for periodic monitoring of kidney function in allopurinol-treated subjects.

In summary, the present study demonstrates the broad efficacy of untargeted plasma metabolite profiling to render a systems biology perspective on the consequences of XOR enzyme inactivation, resulting from either gene deletion or pharmacological inactivation. This comprehensive perspective goes well beyond providing molecular identities of primary XOR substrates and products, by additionally informing on the activity of pathways that are linked to these molecules and the functional integration of XOR with more distal metabolic pathways and physiological systems. Untargeted metabolite profiling provides an unprecedented tool to illuminate the often obscure in vivo push-pull connectivity of metabolic pathways and systemic consequences of pathway perturbations.

Materials and Methods

Animals and Reagents

The animal study protocol was in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the New York Medical College Institutional Animal Care and Use Committee. XOR+/- mice were obtained from Dr. Toren Finkel (NIH/NHLBI) or purchased from an NIH breeding facility and bred locally. Homozygous XOR knockout mice were obtained by screening progeny born to heterozygous parents, followed by PCR amplification using RedTaq ReadyMix PCR reaction mix (Sigma, St Louis, MO) as described in Supplementary Information Legends (Text S1). LC-MS grade acetonitrile (ACN) and ddH2O were purchased from Fischer Scientific, OmniTrace glacial acetic acid and formic acid from EMD Chemicals. All other chemicals and standards were obtained from Sigma Aldrich in the best available grade.

Sample Preparation for Metabolite Profiling

Plasma samples were diluted 1:25 with 70% ACN in ddH2O containing 0.2% acetic acid. The diluted samples were briefly vortexed and centrifuged for 5 min at 16,000×g to pellet precipitated proteins. For aqueous normal phase (ANP) chromatography [15], sample supernatants were directly transferred to autosampler vials for analysis by HPLC-MS. For reversed phase (RP) chromatographic separation, supernatants were dried down under vacuum, then resuspended with 5% ACN in ddH2O containing 0.1% formic acid before analysis by HPLC/MS.

LC-MS and LC-MS/MS Platforms for Metabolite Profiling

Untargeted metabolite profiling was performed using both ANP and RP chromatographic separations (for resolution of polar and nonpolar compounds, respectively), dual spray electrospray ionization and high resolution accurate mass determination using a time-of-flight (TOF) mass spectrometer. Notably, LC-MS produces a continuous multidimensional data matrix, consisting of retention time, mass to charge ratio, and ion abundance.

The LC system comprised a Cogent Diamond Hydride™ (ANP) column (2.1×150 mm, 3.5 μm particle size; Microlab Technology Corp, Eatontown, NJ), a Zorbax SB-AQ (RP) column (2.1×100 mm, 1.9 μm particle size, Agilent Technologies, Santa Clara, CA), and a Model 1200 Rapid Resolution LC system consisting of a binary pump, on-line degasser, thermostatted dual 54-well plate autosampler and a thermostatted column compartment (Agilent Technologies, Santa Clara, CA). A precolumn replacement filter frit (0.5 μm, Upchurch Scientific, Oak Harbor, WA) and rapid resolution cartridge (Eclipse XDB-C8, Agilent technologies) were placed in front of the ANP and RP columns, respectively, to prevent column clogging. The LC flow was coupled to an Agilent model 6250 accurate mass time-of-flight (TOF) mass spectrometer, equipped with dual spray electrospray ionization (ESI) source. A separate isocratic pump was used deliver an internal reference mass solution (ions m/z 121.0509 and 922.0093) to the second ESI source for continuous mass calibration during sample analysis. An Agilent 6538 UHD Accurate Mass Q-TOF with same ANP and RP platform was used to conduct fragmentation analysis for confident molecular identification. LC parameters were set as follows for ANP separation: 5 μl injection volume, 0.4 ml/min mobile phase flow rate, 25°C column temperature and 5°C autosampler temperature. The mobile phase consisted of 0.2% acetic acid in ddH2O (solvent A) and 0.2% acetic acid in ACN (solvent B). Gradient steps were applied as follows: 0–2 min, 85% B; 2–3 min, to 80% B; 3–5 min, 80% B; 5–6 min, to 75% B; 6–7 min, 75%; 7–8 min, to 70% B; 8–9 min, 70% B; 9–10 min, to 50% B; 10–11 min, 50% B, 11.0–11.1 min, 20% B, 11.1–14 min, 20% B; 14–14.1 min, 5% B; 14.1–24 min, 5% B; 24–24.1 min, 85% B and 24–34 min, 85% B. Both positive and negative mass spectra were acquired in 2 GHz (extended dynamic range) mode with 1.41 spectra/sec sampled over a mass/charge range of 50–1000 Daltons. The TOF capillary voltage was set at -4000 V for positive ions and 3500 V for negative ions with the fragmentor set to 175 V. The nebulizer pressure was 35 psi and the nitrogen drying gas was 250°C, delivered at a flow rate of 12 l/min. Data was saved in centroid mode using Agilent MassHunter Workstation Data acquisition Software (revision B346). For RP separation, the mobile phase consisted of 0.1% formic acid in H2O (solvent A) or ACN (solvent B). The gradient was as follows: 0–2 min, 5% B; 2–
17 min, 98% B; 17.1–27 min, 98% B; 27.1–37 min, 5% B. Other LC and TOF parameters used for RP chromatography were the same as for ANP. To minimize potential salt and other contaminants in the ESI source, a time segment was set for both ANP and RP positive and negative acquisitions that directed the first 0.2 ml of column elute to waste.

Data Processing and Analysis

Raw data files were processed using Agilent MassHunter Qualitative Analysis Software (version B.34.6). Untargeted molecular feature extraction (MFE) [16,60] generates features (compounds) based on the elution profile of identical mass and retention times, within a defined mass accuracy (<5 ppm). These features are further grouped into one or more “compounds” based on their isotope pattern, the formation of dimer, adduct ions (e.g. H+, Na+, K+ for positive mode and H--, CH$_3$COO--, HCOO$^-$ and Cl$^-$ for negative ion mode) and common neutral losses of H$_2$O and NH$_3$. Compounds/features with absolute peak heights of 1000 or greater were selected and stored as compound exchange format (CEF) files for feature alignment, data processing and multivariate statistical analysis in Mass Profiler Professional (Agilent Technology, MPP, version B2.02). Each aligned mass was associated with its neutral mass, ion intensity and retention time. The aligned data were filtered, considering only features that were detected in all biological replicates from at least one sample group. To complement the untargeted MFE findings and reduce false-positive and false-negative detection rates, we further conducted recursive analysis, wherein a composite list of ions found by MFE were targeted for re-extraction against raw data for all potential ion species (isotopes, adducts, dimers and trimers). The recursive CEF files were re-imported into MPP to provide a higher confidence analysis. Notably, recursive analysis is effective for finding missing features that would otherwise result in overlooking actual resident metabolites by subsequent statistical analysis.

Statistical Analysis

MPP was used to provide a multivariate statistical platform for comparative metabolite profiling. Principal component analysis (PCA) and hierarchical cluster analysis (HCA) are two unsupervised pattern recognition algorithms used to examine data sets for expected and unexpected clusters, including the presence of outliers without prior sample grouping information. Aligned features detected in all biological replicates of at least one group were directly applied for a 3-dimensional visualization of the data. One-way analysis of variance (ANOVA) was applied to find metabolites showing statistical differences across groups. The Benjamini-Hochberg correction was applied to adjust for false-positive discovery, arising from multiple testing of p-values (adjusted for predicted p<0.05).

Differentially-expressed Metabolite Identification

A critical step in metabolite profiling is identification of unknown metabolites. Differential metabolites with fold changes greater than 2, compared to WT, were initially searched against an in-lab annotated METLIN Personal Metabolite Database (Agilent Technologies), based on accurate monoisotopic neutral masses (<5 ppm). A molecular formula generator (MFG) algorithm in MPP was used to generate and score empirical molecular formulae based on a weighted consideration of monoisotopic mass accuracy, isotope abundance ratios, and spacing between isotope peaks. Notably, MFG imposes additional constraints on the list of candidate molecular formulas detected by a METLIN database search. A putative compound ID was tentatively assigned when METLIN and MFG concurred for a given candidate. Tentatively assigned compounds were verified based on a match of LC retention time and/or MS/MS fragmentation patterns to pure molecular standards. Fragmentation pattern matches were performed for the identification of positional isomers that were unresolved by HPLC and for relatively high molecular weight compounds (600–1,000 Da), where “hits” based on MFG alone can exceed 100 and thus preclude confident molecular identification if considered alone.

Kidney Histology and Assays

Kidneys (from 3–5 mice for each xor genotype) were fixed in 4% paraformaldehyde and embedded in paraffin. Sections (3–4 microns) were stained with hematoxylin/eosin (H&E), periodic acid-Schiff reagents (PAS) and Mason’s trichrome for routine histology. Xanthine and hypoxanthine crystals were visualized by darkfield and polarized light microscopy.

Supporting Information

Figure S1 Box-whisker plot depicting metabolites that change significantly in xor KO vs. WT mice (p<0.05), but not significantly different in WTA (allopurinol-treated) vs. WT mice. The bottom and top of the box denote the 25th and 75th percentile of the ion intensity. The whiskers represent the maximum and minimum of the data. The median and mean are represented as solid and dashed lines, respectively, within each box. (TIFF)

Figure S2 Curated network, depicting the global metabolic consequences of XOR gene deletion. Pathways are plotted using PathVisio (http://pathvisio.org/), a non-proprietary online access tool for displaying and editing biological pathways. Metabolic linkages are from KEGG pathway maps (http://www.genome.jp/kegg-bin/show_organism?menu_type= pathway_maps&org=mmu) and presented using the following nomenclature to denote biological entities: gene products, black font in a black box; metabolites, blue font in a blue box. The pathway is further annotated with KEGG IDs of metabolites and the Entrez gene IDs of gene products. Observed XOR-knockout associated changes in metabolic expression are quantified as Log$_2$ fold-change, relative to XOR wildtype control, and denoted in green for molecules with levels that are upregulated and red for molecules that are downregulated. Metabolites without annotated fold-changes were either undetected by LC-MS or exhibited no significant change from control levels. (TIFF)

Text S1 PCR screening of murine xor genotypes. (DOCX)

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Author Contributions

Conceived and designed the experiments: QC HP MG PC SF SG. Performed the experiments: QC PC. Analyzed the data: PC MG SG. Contributed reagents/materials/analysis tools: SF. Wrote the paper: QC MG SG.
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