Mapping Proteome-wide Targets of Glyphosate in Mice

Graphical Abstract

Highlights

- Mechanisms of toxicity for the herbicide glyphosate are not well understood
- We used activity-based protein profiling to look for glyphosate targets in mice
- Glyphosate is metabolized to glyoxylate and reacts with cysteines on liver proteins
- Glyoxylate inhibits fatty acid oxidation and glyphosate increases liver fat

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In Brief
Ford et al. show that glyphosate is metabolized to reactive metabolites that inhibit fatty acid oxidation and increase liver triglyceride and cholesteryl ester levels.
Mapping Proteome-wide Targets of Glyphosate in Mice

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INTRODUCTION

Glyphosate, the active ingredient in the herbicide Roundup, is one of the most widely used pesticides in agriculture and home garden use. Whether glyphosate causes any mammalian toxicity remains highly controversial. While many studies have associated glyphosate with numerous adverse health effects, the mechanisms underlying glyphosate toxicity in mammals remain poorly understood. Here, we used activity-based protein profiling to map glyphosate targets in mice. We show that glyphosate at high doses can be metabolized in vivo to reactive metabolites such as glyoxylate and react with cysteines across many proteins in mouse liver. We show that glyoxylate inhibits liver fatty acid oxidation enzymes and glyphosate treatment in mice increases the levels of triglycerides and cholesteryl esters, likely resulting from diversion of fatty acids away from oxidation and toward other lipid pathways. Our study highlights the utility of using chemoproteomics to identify novel toxicological mechanisms of environmental chemicals such as glyphosate.

SUMMARY

Glyphosate, the active ingredient in the herbicide Roundup, is one of the most widely used pesticides in agriculture and home garden use, with 180–185 million pounds used in the United States in 2007 (Grube et al., 2011). Glyphosate is also one of the most controversial herbicides, due to major disagreements about its safety and toxicity (Cressey, 2015; Monsanto, 2015). Many studies have associated glyphosate exposure with various adverse health effects (El-Shenawy, 2009; Greim et al., 2015; Mesnage et al., 2015; Portier et al., 2016; Samsel and Seneff, 2015). However, many of these approaches have been largely correlative and have likely still missed subtler or indirect pathological effects that may arise from long-term exposures. We believe that understanding the direct chemical-protein interactions of glyphosate or its metabolites will inform our understanding of downstream molecular, metabolic, and pathophysiological effects, providing a more direct approach toward understanding toxicological mechanisms of this widely used pesticide. In this study, we have mapped glyphosate metabolism, targets, and downstream metabolic consequences in vivo in mice toward better understanding the actions of glyphosate in complex biological systems.

RESULTS AND DISCUSSION

Of concern is whether glyphosate may be biotransformed into electrophilic metabolites, which may in turn react with nucleophilic amino acid hotspots on proteins such as cysteines and lysines, which may cause disruptions in protein biochemistry, such as enzyme catalysis, post-translational regulation, redox balance, metal binding, and protein-protein interactions. Glyphosate has been reported to be metabolized by soil microbes and possibly in mammals to aminomethylphosphonic acid (AMPA) and the reactive metabolite glyoxylate (Samsel and Seneff, 2015). Glyoxylate is an aldehyde known to react with nucleophilic amino acids on protein targets, such as cysteines, lysines, and arginines (Gohre et al., 1987; Schuette, 1998).

To determine whether glyphosate is potentially biotransformed into glyoxylate in mammals, we administered isotopic [13C/15N] glyphosate to mice at a high dose of 200 mg/kg intraperitoneally (i.p.), once per day over 7 days, and measured isotopically labeled [13C/15N]glyoxylate, [15N]AMPA, and [13C]glyoxylate levels ex vivo in mouse liver using single-reaction monitoring (SRM)-based liquid chromatography-mass spectrometry (LC-MS/MS) derived from fragmentation and retention times of standards for each chemical. We acknowledge that these doses are much higher than exposure levels encountered by the public, but toxicological testing studies with pesticides are oftentimes
performed at maximum tolerated doses. Despite previous reports claiming that glyphosate is largely not metabolized in vivo (Williams et al., 2000), we show significant formation of isotopic AMPA and glyoxylate in livers from \([13C/15N]\) glyphosate-treated mice (Figure 1A). The level of glyoxylate formed is approximately 4% of glyphosate levels detected in the liver. Glyoxylate is also produced through various metabolic pathways in mammals, such as glycine degradation (Wang et al., 2013). We also treated mice with nonisotopic glyphosate (200 mg/kg i.p., once per day over 7 days) and show that glyphosate treatment significantly increases glyoxylate levels by \(\sim 2\)-fold above endogenously generated levels at the doses used in this study (Figure 1B).

We postulated that the observed heightened levels of glyoxylate would lead to more glyoxylate reactivity with susceptible nucleophilic residues on proteins in vivo (Williams et al., 2000). When used in a competitive manner, reactive chemicals can be competed against reactivity-based probes to map their proteome-wide reactivity (Counihan et al., 2015; Roberts et al., 2016). First, we performed gel-based ABPP studies in which we determined general cysteine reactivity in mice treated with vehicle or glyphosate by labeling mouse liver proteomes with the cysteine-reactive iodoacetamide-alkyne (IAyne) probe, followed by appendage of rhodamine-azide by copper-catalyzed click chemistry and in-gel fluorescence analysis. We show that in vivo glyphosate treatment selectively reduced cysteine reactivity of several protein targets in mouse liver (Figure 2B; Figure S1). To determine the identity of these targets, we next performed an ABPP proteomic experiment, in which we enriched IAyne-labeled proteins from vehicle- and glyphosate-treated mouse liver proteomes through biotin-conjugation to probe-labeled proteins, avidin enrichment, and proteomic analysis. Of 340 IAyne-enriched proteins, we observed 51 protein targets that showed significantly less IAyne enrichment in glyphosate-treated mouse livers, compared with vehicle-treated controls, indicating that these targets possessed cysteines that were bound by reactive metabolites of glyphosate (Figure 2C; Table S1).

To gain an in-depth understanding of which specific cysteines on proteins were particularly susceptible to in vivo reactive...
A. In vivo competitive isoTOP-ABPP to map proteome-wide cysteine reactivity of glyphosate in mouse liver

- Vehicle-treated control
- Liver proteome from vehicle-treated mice
- Graph showing light/heavy ratio
  - Light/heavy ratio = 1
  - Light/heavy > 1
- HKSAC*SYP (protein A - not inhibited) and YC*STKYGLP (protein B - inhibited)

B. In vivo cysteine-reactivity in mouse liver after glyphosate treatment

Graph showing spectral counts for glyphosate and control

C. ABPP analysis of IAyne labeled protein targets from glyphosate-treated mouse livers

Graph showing modified peptides from control group and modified peptides from glyphosate-treated group

D. isoTOP-ABPP analysis of cysteine-reactivity in glyphosate-treated mouse livers

Graph showing light/heavy ratio against peptides

(legend on next page)
metabolites of glyphosate, we performed isotopic tandem orthogonal proteolysis-enabled ABPP (isoTOP-ABPP) to map proteome-wide cysteine reactivity of glyphosate and its metabolites in vivo in mouse liver (Weerapanap et al., 2010) (Figure 2A). We labeled in vivo vehicle- and glyphosate-treated mouse liver proteomes with IAyne, followed by linking an isotopically light (vehicle) or heavy (glyphosate-treated) tobacco etch virus (TEV) recognition site-bearing biotin handle by click chemistry, combining the vehicle and glyphosate proteomes together in a 1:1 ratio, enriching IAyne-labeled proteins by biotin pull-down, tryptically digesting enriched proteins, and subsequently releasing the probe-modified tryptic peptides by TEV protease digestion. We identified >3,500 peptides bearing light or heavy IAyne-modified cysteines (Table S1). We quantitatively compared and interpreted only those peptides that were found in at least two of the three biological replicates. Among these resulting 320 cysteine-modified peptides, 190 showed >1.5, 67 showed >2, and 20 showed >3 light to heavy ratios (Figures 2D and 3A; Table S1). To determine whether these changes in ratios were dependent on changes in protein expression, we also performed standard proteomic profiling of vehicle- and glyphosate-treated liver proteomes. While 2810007J24Rik and Selenb2 protein expression was significantly lower in glyphosate-treated mouse livers, we show that most of the protein targets that showed >3 light to heavy ratios did not show reduced protein expression, indicating that these targets are likely to be direct targets of glyphosate metabolites (Table S1). Among the top 21 modified peptides showing >3 light to heavy ratios, two of these cysteines corresponded to an annotated catalytic cysteine on Acaaa1b (C123) and Aldh9a1 (C312) and one of these cysteines is an annotated glutathionylated site (C69) on Fabp1 (highlighted in red in Figure 3A), indicating that the function of these enzymes may be impaired by glyphosate or its metabolites in vivo (Chevillard et al., 2004; Dörmann et al., 1993; Riveros-Rosas et al., 2013). Acaaa1b is a thiolase involved in peroxisomal fatty acid oxidation (Chevillard et al., 2004; Fidaloe et al., 2011). Aldh9a1 is an aldehyde dehydrogenase involved in the dehydrogenation of γ-amino butyraldehyde to the neurotransmitter γ-amino butyric acid (Lin et al., 1996). Fabp1 is a cytosolic fatty acid binding protein involved in fatty acid transport and is a central regulator of whole-body metabolic control (Thumser et al., 2014).

We also mapped lysine reactivity using the previously described dichlorotriazine-alkyne (DCTyne) probe (Shannon et al., 2014). In mapping lysine reactivity with isoTOP-ABPP methods, we also identified three additional lysines annotated as known succinylation or acetylation sites on Hsd17b10 (K222), Cps1 (K1269), and Hbb-b1 (K145) that also showed light to heavy ratios >3 (Figure S2).

Mining deeper into our isoTOP-ABPP data, we also noticed that the peptides bearing catalytic cysteines for several thiolase family members also showed light to heavy ratios >1.5, including C32 of the mitochondrial fatty acid oxidation enzyme Acaa2, C94 of the peroxisomal fatty acid oxidation enzyme Scp2, and C92 of the cytosolic acetyl CoA acetyltransferase (Acat2) involved in biosynthesis of ketone bodies such as acetocetyl-CoA (Figure 3B). As Acaaa2, Acat2, and Scp2 protein expression was not significantly reduced upon glyphosate treatment (Table S1), we interpret these data to indicate that these cysteines are directly modified by glyphosate metabolites. We further show that glyoxylate displaces IAyne labeling of pure Acaaa1b, Acaaa2, and Scp2 in vitro and that glyoxylate also inhibits thiolase activities of Acaaa1b and Acaaa2 (Figures 3C and 3D). In addition, we performed isoTOP-ABPP analysis on in vitro glyoxylate cysteine reactivity in mouse liver proteomes and show that glyoxylate shows very similar reactivity signatures to those observed with in vivo glyphosate treatment, where we show that 141 of the 190 in vivo targets of glyphosate (74%) show >1.5 light to heavy ratios. This signature includes Acaaa1b, Acaaa2, and Scp2, which show >1.5 light to heavy ratios for nearly every probe-labeled site on these proteins for both in vitro gly oxylate and in vivo glyphosate treatments (Figure S3; Table S1).

Interestingly, Acaaa1b, Acaaa2, and Scp2 are all involved in mitochondrial or peroxisomal oxidation of long-chain and branched-chain fatty acids (Haapalainen et al., 2006). Genetic deficiencies in these thiolases or in peroxisomal or mitochondrial fatty acid oxidation pathways have been shown to cause liver dysfunction; lipid dysregulation in the form of elevated triacylglycerols, ceramides, and sterols; and hepatic steatosis, likely because the fatty acids that are not oxidized are diverted into other lipid metabolism pathways (Kim et al., 2014; Klipsic et al., 2015; Lee et al., 2016; Mizuno et al., 2013; Wanders et al., 2015).

We hypothesized that labeling of the catalytic cysteines on several thiolases involved in fatty acid oxidation would lead to the inhibition of these targets, impaired fatty acid oxidation, and diversion of fatty acids into other lipid pathways, including...
Figure 3. In Vivo Glyphosate Targets and Biochemical Changes in Mouse Liver

A) Proteins from specific peptides that showed >3 light to heavy ratios from isoTOP-ABPP analysis of mouse livers from vehicle (light)- or glyphosate (heavy) (200 mg/kg i.p., once per day over 7 days)-treated mice. Specific cysteines labeled by IAyne, their corresponding tryptic peptide sequences and sites of glutathionylation (for Fabp1).

B) In vivo cysteine reactivity of thiolases in glyphosate-treated mouse livers

C) validation of thiolase targets glyoxylate

D) thiolase activity

E) lipidomic alterations in mouse livers from glyphosate-treated mice

F) isotopic [13C]Palmitate tracing in HEPG2 cells

G) glyphosate metabolite glyoxylate inhibits fatty acid oxidation causing diversion of fatty acids toward other lipid pathways
triglycerides, sterols, and ceramides. We next performed a lipidomic profiling experiment in which we quantitatively measured the levels of 192 lipid and sterol species in vehicle- compared with glyphosate-treated mouse livers. We identified 62 distinct lipid species whose levels were significantly altered upon glyphosate treatment (Figure 3E, Table S2). These changes included elevations in several neutral lipids, including monoacylglycerols (MAGs), diacylglycerols (DAGs), and triacylglycerols (TAGs). We also observed increases in cholesterol and cholesteryl olate levels as well as other lipid classes. Our data reveal that glyphosate treatment at high doses causes major lipid dysregulation, including increases in fat storage and cholesteryl esters in mouse liver (Figure 3E, Table S2). To further confirm our hypothesis, we also treated HEPG2 hepatocytes with glyoxylate and traced the fate of exogenously added isotopically labeled [13C] palmitate and show that fatty acids are incorporated more into [13C] palmitoyl carnitines (C16:0 AC) and [13C] triacylglycerols (C16:0/C18:1/C16:0 TAG) (Figure 3F). These data are all consistent with our premise that inhibition of fatty acid oxidation enzymes by the glyphosate metabolite glyoxylate is associated with diversion of fatty acids into other lipid metabolism pathways.

We show here using both chemoproteomic and metabolomics approaches that glyphosate may be metabolized to reactive metabolites such as glyoxylate, which may react with and inhibit many cysteine-reactive protein targets in vivo in mouse liver, including several fatty acid oxidation enzymes, which may be associated with elevations in liver triacylglycerols, cholesteryl esters, and other lipid species (Figure 3G). We caution that the doses used in this study are much higher than any exposure levels encountered by the public and that further studies using lower doses in more relevant exposure paradigms will be necessary to fully evaluate these findings. Our data nonetheless show that glyphosate may be biotransformed to reactive metabolites that broadly react with key cysteines and lysines across many proteins in vivo, several of which are likely to cause dysfunction of those proteins and downstream physiological effects. Furthermore, while we identify glyoxylate as one possible metabolite of glyphosate that could be reactive, there may be additional reactive metabolites, which may explain the reactivity profiles observed in this study (Gohre et al., 1987). We also do not yet understand the tissues, cell types, and enzymes responsible for glyphosate metabolism and we cannot necessarily rule out the gut microbiome as a source for glyphosate metabolism into glyoxylate.

Nonetheless, our study provides much-needed mechanistic insights into the potential toxicities and toxicological mechanisms associated with glyphosate. Our data here show that in vivo glyphosate exposure may lead to generation of reactive metabolites such as glyoxylate, which may in turn inhibit fatty acid oxidation enzymes. We also show that glyphosate treatment at high doses is associated with heightened levels of triglycerides and cholesteryl esters and may potentially lead to corresponding metabolic disorders. Our results highlight the utility of using chemoproteomic platforms such as ABPP to map the proteome-wide reactivity and targets of environmental chemicals toward understanding their mechanisms of toxicity.

**SIGNIFICANCE**

In this study, we have used chemoproteomic and metabolomic platforms to map the direct targets and downstream metabolic consequences of glyphosate and its metabolites in vivo in mice, toward better understanding the potential toxicological mechanisms of this active ingredient found in the very widely used herbicide Roundup. We show that glyphosate is metabolized in vivo to a reactive metabolite, glyoxylate, at high doses in mice, where it reacts with many protein targets. We show that fatty acid oxidation enzymes are inhibited by glyoxylate and that glyphosate treatment in mice leads to elevated levels of fat and cholesteryl esters in the liver.

**EXPERIMENTAL PROCEDURES**

**Mice**

Male C57BL/6 mice (6–8 weeks old) were acutely (2 hr) or subacutely (7 days) exposed by i.p. injection to 200 mg/kg glyphosate (Sigma 45521) in a vehicle of PBS (10 μL/g mouse weight). Following exposure, mice were sacrificed by cervical dislocation, and liver and serum were immediately removed and flash frozen in liquid nitrogen. Animal experiments were conducted in accordance with the guidelines of the Institutional Animal Care and Use Committee of the University of California, Berkeley.

**Processing of Mouse Liver Proteomes**

Tissues were homogenized in PBS, followed by a 1,000 g centrifugation of the homogenate. The resulting supernatant was collected and used for subsequent assays. Protein concentrations were determined by BCA protein assay (Pierce).

**Activity Assays**

Enzymatic activity of Acaa1b and Acaa2 was performed using the Fluorometric Acetyltransferase Activity Assay Kit (Abcam ab204536). The assay was performed per the protocol with 0.4 μg of pure protein, 3 mM glyoxylate, and 100 mM acetoacetyl-CoA (Sigma A1625); fluorescence was measured at 380/520 ex/em on a SpectraMax i3x detection platform.

**Proteomic Analysis**

For ABPP and isoTOP-ABPP analysis, we performed studies as previously described (Medina-Cleghorn et al., 2015; Weerapana et al., 2010). Detailed procedures are described in the Supplemental Experimental Procedures.
Metabolomic Profiling
Metabolomic profiling was performed as previously described (Benjamin et al., 2013; Louie et al., 2016). Detailed methods are described in the Supplemental Experimental Procedures.

SUPPLEMENTAL INFORMATION
Supplemental Information includes Supplemental Experimental Procedures, three figures, and two tables and can be found with this article online at http://dx.doi.org/10.1016/j.chembiol.2016.12.013.

AUTHOR CONTRIBUTIONS
B.F. and D.K.N. performed experiments, interpreted data, and wrote the paper. L.A.B. and L.G.P. performed experiments; R.P. provided bioinformatics support for analyzing the quantitative proteomics data.

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