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Glyphosate Substitution for Glycine During Protein Synthesis as a Causal Factor in Mesoamerican Nephropathy

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Abstract

Mesoamerican Nephropathy (MeN), also known as Chronic Kidney Disease of unknown etiology (CKDu), is an unusual form of kidney disease affecting agricultural workers in Central America. Its prevalence is alarmingly high among young male sugarcane workers in Nicaragua and El Salvador. The absence of known etiologies for CKD, such as hypertension and diabetes, has led researchers to explore a number of potential risk factors, though none adequately explain the timing and epidemic nature of the disease. In this paper, we explore the idea that glyphosate, an herbicide routinely used on sugarcane, could play a significant causal role in MeN, mediated by its property as an analogue of the coding amino acid glycine. Glyphosate is a glycine molecule with a methyl phosphonyl group attached to its nitrogen atom. Its substitution in place of glycine could disrupt multiple proteins critical for kidney health. Here, we first present prior evidence from the research literature that glyphosate may be substituting erroneously for glycine. In particular, multiple species of both bacteria and plants have mutated to remove a highly conserved glycine residue in the enzyme in the shikimate pathway that is disrupted by glyphosate, and this mutation has caused the enzyme to be completely insensitive to glyphosate. We have identified multiple proteins with key roles related to kidney function, whose disruption by glyphosate substitution for critical glycine residues could explain most of the unique features of MeN. Specifically, glycine substitution in aquaporin, chloride channels, cytochrome C oxidase and collagen, among others, could contribute to dehydration, increased urinary acidification, renal fibrosis, rhabdomyolysis and mitochondrial dysfunction. While the hypothesis that glyphosate could be disrupting protein synthesis is not yet proven, it is remarkable how well it explains multiple features of MeN. Investigations to verify whether glyphosate is in fact disrupting protein synthesis are urgently needed.

Keywords: Chronic Kidney Disease of unknown etiology (CKDu); Mesoamerican nephropathy (MeN); Glyphosate; Glycine; Amino acid analogue

Introduction

The remarkably strong correlations between the nearly exponential growth in glyphosate usage on core crops in the U.S. over the past two decades and the alarming rise in a long list of debilitating chronic diseases can only be explained biologically if glyphosate has a unique mechanism of toxicity that affects multiple aspects of cellular metabolism and homeostasis [1-3]. One possibility is that glyphosate, acting as an amino acid analogue of glycine, can erroneously become incorporated into proteins in place of glycine [4-7]. This would lead to a cumulative and insidious toxic effect. Glyphosate is a glycine molecule with a methyl phosphonyl group attached to its nitrogen atom. Proline is a coding amino acid that, like glyphosate, has an additional carbon bond on the nitrogen atom, but this does not preclude its incorporation into a peptide chain, demonstrating that glyphosate could do the same. Because its core structure is a glycine molecule, glyphosate can potentially be misinterpreted as glycine, based on an apparent match to the DNA code for glycine.

Protein synthesis is inherently an errorful process. After catastrophic mistakes are detected through misfolding, there is a mechanism to target them for clearance and reassembly [8,9]. A metatranscriptome study on glyphosate exposure to the rhizosphere of glyphosate-tolerant corn revealed a significant increase in the expression of proteins involved in both protein synthesis and protein degradation [5,10]. This can be expected if glyphosate is increasing the rate of protein misfolding due to glycine substitution. This would necessitate wasted energy involved in disassembly and reassembly of proteins until a functional version is finally produced. Another study from 2017 on the effects of low-dose glyphosate on the soil filamentous fungus Aspergillus nidulans was consistent with this result. In addition to upregulation of multiple detoxification pathways, protein synthesis and amino acid metabolism were both upregulated [11].

Perhaps the most compelling evidence that glycine substitution is happening comes from the effect glyphosate has on the enzyme 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase (EPSPS) in the shikimate pathway in plants and microbes [12-14]. This is alleged to be the main toxic effect of glyphosate on plants. Multiple species of both plants and microbes have independently developed resistance to glyphosate by altering the genetic code to replace a critical glycine reside at the active site for phosphoenol pyruvate (PEP) with alanine [6,7]. A study on Eschericia coli mutants showed that this substitution resulted in complete insensitivity to glyphosate suppression even at very high concentrations [13]. An identical mutation in another microbial species is the basis for the genetically engineered glyphosate resistance in core crops such as corn and soy [15].

As described in a companion paper, glyphosate's application to sugarcane crops increases sugar production, due to glyphosate's suppression of two enzymes where PEP is a substrate, EPSPS and PEP

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carboxylase (PEPC). PEPC, like EPSPS, has an essential glycine residue that, if substituted for glyphosate, would strongly suppress enzyme activity [16]. PEP accumulates due to blockage of these pathways and this leads to excess accumulation of fructose, which is a precursor to PEP.

The idea that glyphosate might be substituting for glycine was first introduced in a paper by Samsel and Seneff published in 2016 [4]. The research literature was systematically searched to identify proteins with highly conserved glycine residues that play essential roles. Remarkably, multiple diseases and conditions whose incidence in the population is rising dramatically in step with the rise in glyphosate usage can each be linked to specific proteins with highly essential glycines. Three subsequent papers each focused more narrowly to explain a link between glyphosate and ALS, gout and autoimmune disease [5-7]. ALS was a particularly striking example because many genetic mutations that are linked to familial ALS are either substitutions for highly conserved glycines or alterations of other amino acids within glycine rich regions. Gout is one of the conditions whose incidence is rising in the population in the past two decades, in step with the dramatic increase in the use of glyphosate on core crops.

Although glyphosate is a synthetic molecule never produced by any living organism, there are several naturally produced amino acid analogues that cause debilitating disease by misincorporating into proteins in place of an analogous coding amino acid [4]. In fact, the herbicide glufosinate is a naturally produced amino acid analogue of glutamate. L-azetidine-2-carboxylic acid (Aze) misincorporates in place of proline causing multiple sclerosis, and N-β-methylamino-l-alanine (BMAA) misincorporates in place of serine, causing an ALS-like condition [17,18]. BMAA, produced by cyanobacteria, has been linked to a condition resembling ALS and Parkinson's disease that reached epidemic proportions in Guam following World War II due to exposure from cycad flour, through its misincorporation into proteins in place of serine [18]. Notably, any BMAA that is misincorporated into proteins may be missed in analysis without sufficient proteolysis. Ince et al. wrote: "When the insoluble, protein-containing fraction following TCA (trichloroacetic acid) extraction is further hydrolysed to release BMAA from protein, there is a further pool of protein-bound BMAA that is present in a ratio of between 60:1 and 120:1 compared with the pool of free BMAA" [19].

A similar problem can be expected when trying to detect glyphosate that has been misincorporated into proteins. In 2007, DuPont conducted a study on goats exposed to radiolabelled N-nitrosyl glyphosate, in which they found that only 42% of the total radiolabel recovered from muscle tissue could be detected as free glyphosate [4,20]. Extensive proteolysis by pepsin and protease digests recovered only a little more glyphosate signal. One explanation is that glyphosate is incorporated into the peptide sequence and causes it to resist proteolysis, and therefore resist detection.

In researching the literature for this paper, we focused on searching for essential glycine residues in proteins that would be implicated in the specific disease manifestations of MeN. We were richly rewarded, in that we were able to find specific proteins with highly conserved glycine residues whose disruption could explain specific aspects of the disease pathology, as shown in Table 1. For example, defective aquaporin would intensify the effects of dehydration, misfolded collagen and defective matrix metalloproteinase activity could explain the interstitial fibrosis, defective proteins involved in urate transport would contribute to elevated urinary urate, and defects in other proteins predicted from glyphosate substitution for glycine can explain acidic urine, impaired iron uptake, impaired clearance of cellular debris and impaired mitochondrial function to synthesize ATP, contributing to oxidative damage.

Iron Toxicity and Deficiency

Iron, while essential, can also be toxic if it is not properly managed. One of the earliest events in kidney dysfunction is oxidative damage due to free iron [21]. Mislocalized iron has been identified as a critical factor in tubular necrosis. Free iron catalyzes the conversion of hydrogen peroxide to the hydroxyl radical, and it can also form reactive ferryl or perferryl species [22]. These reactive small ions can cause significant damage to lipids, nucleotides, and the DNA backbone [23,24]. Multiple papers have implicated iron specifically as a key factor in tubular cell damage [25-27]. The stress response releases cytokines which activate protein kinases, leading to ferritin degradation and the release of free iron, causing oxidative damage to DNA, lipids, and proteins [28]. While creatinine is a commonly used biomarker for kidney function, it is not a sufficiently sensitive marker for early detection of tubular injury. Researchers and clinicians have identified several novel biomarkers that may be more useful, including interleukin 18 (IL-18), N-acetyl-β-D-glucosaminidase (NAG), and neutrophil gelatinase-associated lipocalin (NGAL). NAG is a lysosomal enzyme which is shed into the urine following proximal tubular epithelial cell injury [29]. IL-18 is a pro-inflammatory cytokine produced by both immune and non-immune cells.

NGAL, a protein that is heavily involved with iron homeostasis, is highly upregulated and released into both plasma and urine following tubular injury. It reflects damage to glomeruli, proximal tubules and distal nephrons [30]. A study on 284 sugarcane workers in seven different job categories found that cane cutters and irrigators had the highest increases in NGAL levels during the harvest season [31]. A glycine-X-tryptophan (GXW) motif is a signature for the lipocalin family, of which NGAL is a member, and these proteins also contain two other highly conserved glycines, so there is a possibility of disruption by glyphosate substitution [32].

NGAL, also known as siderocalin, was first understood to play a role in sequestering iron to prevent iron acquisition by invasive pathogens, through its binding to iron-chelating siderophores secreted by the microbes [33-35]. However, it is now realized that it also acts as an important supplier of iron to the host cell in some cell types, and this is particularly relevant to the kidneys [36]. While most cells acquire iron by capturing iron-loaded transferrin, an alternative novel mechanism to acquire iron is through endocytosis of NGAL carrying an iron-loaded bacterial siderophore as cargo. This strategy is active during fetal organogenesis of the kidney tubules, and it also appears to be important in supplying iron to the adult tubules, especially under environmentally stressful conditions. The mouse ortholog of NGAL, called 24p3, is expressed in the fetal kidney where it delivers iron to nonepithelial mesenchymal cells, inducing their transformation into epithelial tubule cells and thus promoting organogenesis of the kidney tubules [37].

An elegant set of experiments on mice confirmed that NGAL is endocytosed through receptor-mediated processes, and that it can induce cellular apoptosis by depleting the cell of iron and exporting the iron to the external environment. If, on the other hand, prior to endocytosis, it has bound to iron-loaded bacterial siderophores, it can supply the cell with iron and protect it from apoptosis [38]. Thus, it now appears as if NGAL not only deprives pathogens of iron but also supplies the iron it acquires from the pathogen to the host cell. And it

Protein	Essential G	Consequence	References
EPSPS	G100	hyperuricemia	[6,15]
PEPC	terminal G	hyperuricemia	[6,16]
MMP-9	HELGHSLGLXHS motif	interstitial fibrosis	[51]
Collagen	Gxx repeats	interstitial fibrosis	[59]
URAT1	G65	hyperuricosuria and nephropathy	[75]
GLUT9	G216	hyperuricosuria and nephropathy	[74]
Na+/H+ exchanger	G455 & G456	acidic urine	[73]
Aquaporin	G57 & G173	dehydration	[82]
CLCN5	G111	megalin deficiency	[64]
NGAL	GXW motif	impaired iron uptake	[32]
Rbn5	G1273	impaired lysosomes	[70]
SDHAF2	G78	impaired Complex II	[93]

Table 1: Partial list of proteins with highly conserved glycines that can explain many of the features of MeN through glyphosate substitution for these glycines.

can also retrieve iron from a dying cell for repurposing.

Siderophores are remarkably strong iron chelators, binding with an association constant in excess of 10^{50} [39]. This enables siderophores to extract iron from transferrin and lactoferrin. One of the strongest siderophores known is enterobactin, which is produced by gram-negative bacteria such as E. coli and Salmonella [40]. Enterobactin is synthesized from chorismate on a branch from the shikimate pathway [41]. Glyphosate's suppression of EPSPS can be expected to reduce chorismate bioavailability, suppressing enterobactin synthesis, just as it suppresses the synthesis of the aromatic amino acids [15]. It is well established that NGAL binds to enterobactin [34]. Bacillibactin, produced by Bacillus subtilis, is also a product of chorismate. Thus, it can be predicted that glyphosate may impede the ability of epithelial tubular cells to acquire iron through the endocytic uptake of NGAL bound to siderophores such as enterobactin or bacillibactin. Bacterial siderophores have shown promise as a therapeutic agent in ameliorating reperfusion following cardiac ischemia in a dose-dependent manner, and the mechanism is proposed to be protection from oxidative damage from free iron [42]. However, in light of the above discussion, it probably also supplies iron through a safe delivery mechanism to the cardiac cells.

MMPs and Interstitial Fibrosis

High levels of proteinuria are uniquely not a feature of MeN, and this points to tubulointerstitial disease as the initiating toxic mechanism [31]. Interstitial fibrosis in the renal tubules is in fact a core feature [43]. Sugarcane workers showed more interstitial fibrosis and tubular atrophy than other occupational groups. Matrix metalloproteinases (MMPs) are a large class of enzymes that are responsible for the breakdown of extracellular matrix materials, particularly collagen and elastin. It was originally believed that they should be protective against fibrosis, by virtue of their ability to degrade the extracellular matrix in the scar tissue. However, it is now appreciated that they also act as signaling molecules to initiate and sustain kidney fibrosis, in part through inducing epithelial-to-mesenchymal transformation (EMT) of tubular cells and activating resident fibroblasts [44]. MMP-9 is highly expressed in the kidney under stress conditions, and upregulated by TGF-β1 [44]. It is activated under both oxidizing conditions and acidic conditions [45,46]. Invading macrophages secrete MMP-9, which induces a profibrotic transformation of tubular cells.

In vitro studies have shown that hemoglobin and iron are toxic to proximal tubular epithelial cells [47]. Transferrin complexed with free iron causes oxidative damage to proximal tubule cells *in vitro* [48]. Upon exposure to transferrin, inflammatory cells, including both macrophages and T lymphocytes, infiltrate the interstitium and induce excessive deposition of extracellular matrix proteins, forming scar tissue [49]. This can eventually lead to sclerosis of the interstitium and tubular atrophy, a classic feature of Mesoamerican nephropathy. Cytokines induce fibroblast infiltration, and fibroblasts proliferate and secrete additional extracellular matrix proteins. As extracellular matrix protein accumulates, it increases the distance between tubular cells and the capillaries that supply them with oxygen, leading to a hypoxic environment that can cause ischemic injury [50]. How much extracellular matrix actually accumulates depends critically on the balance between rates of production and degradation by MMPs. Scarring could be a result of decreased activity of the proteases that don't keep pace with matrix accumulation.

Collagen is a significant component of the matrix proteins in scar tissue in interstitial disease. MMP-9 is a member of the matrix class of proteolytic enzymes, acting as a collagenase and a gelatinase. Crucially, this class contains a zinc-binding catalytic site containing the HELGHSLGLXHS motif [51]. Notably, this motif contains two glycine residues that could be displaced by glyphosate, disrupting protein function. Furthermore, glyphosate has been shown to chelate zinc, making it unavailable in plants and significantly reducing the amount of zinc that is taken up into the roots and stems [52-55]. In a paper investigating the effects of subchronic oral exposure of rats to glyphosate, the authors reported that glyphosate exhibited toxicity towards both the liver and the kidneys, but that prior zinc supplementation greatly ameliorated the effects [56]. This suggests that glyphosate induces zinc deficiency that impairs the function of zinc-dependent enzymes such as MMP-9. MMP-9 plays a dual role in liver disease similar to its dual role in kidney disease, through its positive role in degrading extracellular matrix and its negative role in inducing further synthesis of extracellular matrix [57].

Collagen itself is highly enriched in glycine, with glycine residues making up 20 to 25 percent of the amino acids in a typical collagen molecule. In fact, glycine must occur at every third residue to allow the structure to fold as a triple helix [58,59]. Glycogen binding to collagen also depends on these glycine repeat triplets [60]. Thus, glyphosate contamination in collagen can be expected to disrupt its crystalline structure, potentially increasing the likelihood of an autoimmune reaction to the exposed, misfolded protein. Glyphosate embedded in collagen would also be expected to increase its resistance to proteolysis.

Thus, both disrupted collagen and disrupted MMP-9, along with zinc deficiency, can all contribute to a pathological state whereby the breakdown of extracellular matrix does not keep pace with its build-up induced by cytokines.

Lipoprotein receptor-related protein 2 (LRP2), also called megalin, is directly involved with capture, internalization and clearance of MMP-9, along with many other small proteins [61]. Megalin also binds strongly to NGAL, so defective megalin can be predicted to also contribute to impaired iron uptake in the tubules [62]. The H^+/Cl- exchange transporter 5 protein (CLCN5) is a chloride ion transporter that is expressed in tubular epithelial cells and that plays a regulatory role in megalin expression. A genetic condition called Dent's disease is associated with mutations in this gene and leads to renal tubular disorders, particularly nephrolithiasis [63]. A case study of a Japanese patient with Dent's disease determined that a mutation in the 333rd codon of CLCN5 caused a substitution error replacing glycine with arginine [64]. Not only did this mutation abolish chloride currents, but it also induced impaired N-glycosylation of the mutant protein, as well as markedly reduced expression of megalin in the proximal tubules. The defective chloride pump leads to a failure to acidify the endosome, and this results in a defect in detaching ligands from megalin, a subsequent defect in lysosomal degradation of endocytosed ligands, and, finally, a failure of megalin to return to the membrane for recycling [65-67]. One consequence would be impaired megalin-mediated clearance of MMP-9, thus aggravating tubular fibrosis.

It should also be noted that a study of various biometrics of MeN noted that serum chloride levels were low in association with the disease, likely due in part to chloride loss through profuse sweating [68]. Low cytosolic chloride disrupts lysosomal acidification, working synergistically with other factors to disturb endocytic recycling [69]. Glyphosate substitution for glycine in Rabenosyn-5 (Rbn5) would also be expected to disrupt tubular receptor-mediated endocytotic trafficking. Rbn5 has a highly conserved glycine residue at location 1273. A genetic mutation replacing this glycine with arginine results in a severe disease profile, associated with impaired endosomal/lysosomal trafficking [70].

Urine Acid pH

The kidney is always engaged in luminal H^+ extrusion because of the need to reclaim bicarbonate. This works against the electrochemical gradient and therefore is costly in terms of ATP consumption. The tubules pump sodium out through the Na/K^+ ATPase pump located in the basolateral membrane, and then rely on the sodium gradient to swap Na^+ for H^+ on the luminal side, using Na^+/H^+ exchangers (NHEs) [71-73]. These important proteins that exchange sodium for hydrogen across lipid bilayers are found universally in prokaryotes, animals and plants [71]. The secretion of protons into the urine results in urine acidification. NHE3 is expressed at the apical (luminal) membrane of the epithelial cells in the proximal tubules, in some of the long thin descending limbs, and in the loop of Henle, and it plays an important role not only for bicarbonate absorption but also volume homeostasis and the absorption of other solutes through transporter coupling [71].

NHEs sense intracellular pH to determine activity level, with activity increasing with increasing acidity. A glycine-rich sequence in transmembrane 11, with the consensus sequence YGGLRGA, has two highly conserved glycine residues (Gly455 and Gly456) that are involved in pH sensing. Substitution of either of these for a bulkier residue leads to an alkaline shift in the response, resulting in an increase in pump activity for a given pH, which, in the case of the tubule, would lead to increased acidification of the urine [73]. Thus, glyphosate substitution for either of these glycine residues would be expected to have a similar effect, and this would cause an increased risk of precipitation of urate crystals, leading to tubular damage. Acid pH might be compounded by excessive urate concentrations potentially induced by glyphosate misincorporation into SLC2A9, which codes for GLUT9, a transporter of both glucose and uric acid. A genetic mutation (G216R) in the SLC2A9 gene leads to severe disease with acute kidney injury in early childhood. This is associated with increased urinary urate due to impaired reuptake in the tubules [74]. A G65W mutation in the urate transporter URAT1 has a similar effect [75].

Aquaporins and NSAIDS

Nocturia is a commonly reported symptom of MeN, and it can be related to impaired reabsorption of water in the nephron [76,77]. In the kidney, approximately 60 to 70% of the filtered sodium and water is reabsorbed in the proximal tubules of the nephron, together with approximately 90% of the filtered bicarbonate [78]. The process is controlled by many regulatory factors, including angiotensin II, endothelin, parathyroid hormone, dopamine, and pH. The main proteins involved in sodium transport are the luminal membrane Na^+/H^+ exchanger and the basolateral Na^+/K^+ ATPase pump. Luminal membrane aquaporin channels control water reabsorption driven by the osmotic gradient. Aquaporins are a suite of integral membrane proteins that form pores in the membranes of cells to facilitate the transport of water through the membrane [79]. At least seven different aquaporins are expressed in the kidneys, where they play a crucial role in the regulation of water balance. Solutes are concentrated in the urine by secreting water through aquaporin channels, returning it to the circulation and therefore conserving water for the body, which is especially important during dehydrating conditions such as excessive perspiration during hard labor in a hot climate. More specifically, aquaporin channels mediate the osmotic water transport across the renal medullary epithelium.

AQP2 activity is regulated by the antidiuretic hormone vasopressin, which increases the number of AQP2 channels in the cell membrane of cells in the collecting ducts to promote water retention [80]. One theory to explain Mesoamerican nephropathy is based on the idea that NSAIDs, taken to suppress aches and pains from muscle overexertion, may cause excessive water loss through the urine during dehydrating conditions through their action on vasopressin. A study on rats demonstrated that NSAIDs decrease AQP2 expression significantly in water-restricted rats [81]. Ibuprofen prevents the increase in AQP2 expression that normally occurs in response to vasopressin signaling following dehydration. We hypothesize that an additional compounding factor is glyphosate substitution for one of the two essential glycine residues in aquaparin. Most of the mammalian aquaporins contain two highly conserved glycine residues: Gly-57 in transmembrane helix (TM) 2 and Gly-173 in TM5 situated at the contact point where the two helices cross in human AQP1 [82]. AQP6 is unusual in that it has asparagine instead of glycine at residue 57, and this completely changes its character such that it becomes an anion channel rather than a water channel. Swapping in glycine for the asparagine converts it into a water channel. This demonstrates that Gly-57 is essential for aquaporins to function as water transporters.

Sodium Potassium Pump and Rhabdomyolysis

The combination of repetitive heat stress, dehydration and strenuous work, viewed as the main risk factors for MeN, chronically activate the renin angiotensin aldosterone system [83]. Both aldosterone and vasopressin cause an increase in the expression of Na^+/K^+ ATPase in cortical collecting duct cells in the kidney [84]. Aldosterone also regulates sodium transport in the proximal tubule via a mineralocorticoid receptor stimulator pathway [85]. Aldosterone

causes an increase in the reabsorption of sodium ions from the tubular fluid back into the blood, while causing a loss of potassium ions into the urine in exchange, leading to hypokalemia. This pathology is exasperated by systemic inflammation due to rhabdomyolysis and uricosuria [86].

Hypokalemia itself can cause rhabdomyolysis through inadequate vasodilation of capillaries perfusing exercising muscle, suppression of glycogen synthesis, and deranged ion transport across muscle cell membranes [87]. Fluid extracted from the circulation into swelling muscles can induce hypotension, a feature of MeN despite overexpression of the renin angiotensin aldosterone system. Renal uptake of myoglobin released from damaged muscles is mediated by the endocytic receptors, megalin and cubilin [88]. As we have argued previously, megalin function may be disrupted by glyphosate, causing impaired clearance of myoglobin in rhabdomyolysis.

Mitochondrial Complex II

In mitochondria isolated from rat livers, five hours after a single intraperitoneal 60 mg/kg dose, glyphosate enhanced the rate of oxygen consumption by 40%, attributable to uncoupling of mitochondrial oxidative phosphorylation [89]. This might be explained in part by glyphosate substitution for one of two highly conserved glycines in cytochrome C oxidase [90]. Another study on isolated rat liver mitochondria exposed to Roundup showed inhibition of Complex II and Complex III mediated by a collapse of the transmembrane electrical potential [91]. Succinate dehydrogenase (SDH) is essential to the function of Complex II in the citric acid cycle and in the electron transport chain. It oxidizes succinate to fumarate, and its enzymatic function depends on attachment of flavinin adenine dinucleotide (FAD), a process termed flavinylation. Genetic mutations in SDH have been linked to renal carcinoma [92].

An assembly factor termed SDH Assembly Factor 2 (SDHAF2), also known as SDH5, binds to the flavoprotein SDH1 to allow FAD entry. A genetic mutation resulting in a substitution of arginine for a highly conserved glycine residue (G78R) in SDHAF2 is a neuroendocrine tumor susceptibility genotype [93]. Three individuals suffering from paraganglioma were found to carry this same identical SNP [94]. Although SDHAF2 shows great diversity among species at the amino acid sequence level, G78 is conserved across plants, animals, and microbes, as shown in Table 2 [93]. There is also a highly conserved glycine residue in SDH1 adjacent to a conserved arginine residue in the C-terminal region that is essential for FAD insertion [93].

Vitamin D Metabolism and Sulfate Deficiency

Glyphosate exposure has been implicated in the Vitamin D deficiency epidemic in the U.S. over the past two decades [95,96]. Vitamin D is activated in a two-step process, where vitamin D3 is converted to 25(OH)-D3 by liver cytochrome P450 (CYP) enzymes and is then converted to the active form, 1,25(OH)-D3 by renal CYP enzymes. Glyphosate has been shown to severely suppress CYP enzymes in rat studies [97]. Another issue is impairment of the uptake and recycling of vitamin D binding protein, which depends on megalin as the receptor [98]. A consequence of impaired megalin-based endocytosis from glyphosate substitution for glycine is disruption of the recycling of vitamin D binding protein, which is essential as a first step in the uptake of 25(OH)-D3 and its subsequent conversion to 1,25(OH)-D3 by CYP enzymes in the renal tubules [98].

Species	Sequence
Mammal	KRGMLENCIL
Fungus	KRGILETDLL
Probacteria	RRGMRELDIS
bryophyte	QRGYLELDLL
Green algae	QRGFLELDIV
Eudicot	QRGFLELDLV
Monocot	QRGFLELDLV

Table 2: Sequence alignment of a peptide sequence within SDHAF2-like proteins from diverse species, showing highly conserved glycine at residue 78. Adapted from Huang and Millar, 2013 [93].

Megalin-mediated endocytosis of receptors and sulfated mucins helps to maintain the acidic environment that supports completion of the endocytosis/receptor recycling process. The renal sodium-sulfate cotransporter, NaS(i)-1 controls serum sulfate levels by promoting sulfate reabsorption in the renal tubules [99,100]. The promoter for this transport protein has a vitamin D responsive element, and thus 1,25(OH)-D3 enhances its transcription. Indeed, vitamin D3 deficient mice exhibit low serum sulfate along with a three-fold increase in renal excretion of sulfate and a 78% decrease in renal NaS(i)-1 [101]. Vitamin D receptor (VDR)-deficient mice also have low serum sulfate and high urinary sulfate, a clear indicator of the important role vitamin D plays in protecting from sulfate loss through the urine [100]. These mice also had a dramatic reduction in skeletal sulfated proteoglycan synthesis, and a reduction in glutathione levels. Since glutathione is an important antioxidant, this impacts the risk of oxidative damage due to inflammation. It may also be the case that impaired arylsulfatase due to glyphosate substitution for glycine results in an inability to detach sulfate from vitamin D sulfate and therefore also interferes with vitamin D activation and sulfate homeostasis [4,102].

Sulfite oxidase is an essential molybdenum-dependent enzyme that converts sulfite to sulfate. Genetically-based sulfite oxidase deficiency results in intractable seizures, microcephaly, and profound mental retardation [103-105]. Elevated serum sulfite is associated with chronic kidney disease, suggesting that a defect in sulfite oxidase may be a feature of this condition. Xanthine oxidase is an enzyme involved in urate synthesis, and it also depends on molybdenum as a cofactor. It stands to reason that excessive expression of xanthine oxidase, as can be expected with high serum urate, might deplete the supply of molybdenum for sulfite oxidase. Glyphosate is well established as a chelator of +2 cations, so it might also play a role in making molybdenum less available for sulfite oxidase [106]. Furthermore, sulfite oxidase contains a heme group, and the synthesis of the pyrrole ring, a core component of heme, depends on aminolevulionic acid synthesis, which is suppressed by glyphosate [105].

Finally, a crucial glycine residue at position 473 in sulfite oxidase is essential for its function. A mutation converting this glycine residue to aspartate results in impairment both in the ability to bind sulfite and in the ability to form a dimer, resulting in a second-order rate constant that is 5 orders of magnitude lower than that of the wild type [106]. Glyphosate substitution for this glycine residue would also severely disable the enzyme. Thus, defective sulfite oxidase is an additional contributory factor to sulfate deficiency, along with the wasting of sulfate through urine due to vitamin D deficiency.

Relation to Barrter Syndrome

Salt absorption, the regulation of divalent mineral cations, and acid-base regulation are all important roles of the thick ascending limb of Henle's loop in the nephron, and a critical protein involved in this regulation is the Na-K-Cl cotransporter, NKCC2 [107]. Barrter syndrome is a genetic condition that is often manifested by many of

Disruption of Metabolites/Processes	Damaging Consequences
Direct injury	Hypotension and hypovolemia; oxidative stress
Hypokalemia	Rhabdomyolysis; hypotension
URAT1; GLUT9	Impaired urate transport; high urinary urate
Aquaporin	Dehydration
Na+/H+ exchangers	Increased urinary acidification; urate crystallization
Collagen	Interstitial fibrosis
Zinc	Impaired MMP-9 function; Kidney fibrosis
Chloride pump	Impaired acidification of endosome
Siderophores	Impaired iron transport
NGAL	Impaired iron homeostasis; iron toxicity
Rbn5	Impaired endosomal/lysosomal trafficking
Megalin recycling	Vitamin D deficiency; impaired endocytosis; impaired iron uptake; impaired myoglobin clearance = rhabdomyolysis
Vitamin D activation	Vitamin D deficiency; Sulfate loss
Mitochondrial Complex II	Reduced ATP production; oxidative stress
Molybdenum	Impaired sulfite oxidase; sulfite toxicity; sulfate deficiency
Chloride transporter NKCC2	Bartter Syndrome
Cytochrome P450 enzymes	Impaired xenobiotic clearance

Table 3: Summary of various disruptions of important metabolites and processes that can be expected to be caused by glyphosate exposure, if glyphosate can substitute for glycine during protein synthesis, with associated pathologies.

the features that are characteristic of MeN, including hypotension, low serum potassium and chloride, metabolic alkalosis, polyuria and elevated aldosterone / activated renin system. Many patients with Barrter syndrome have genetic defects in NKCC2. A single mutation in exon 4 of NKCC2 results in a G224D substitution expressed as Barrter syndrome [108]. The replacement of this glycine residue at the site where chloride enters with aspartate introduces a negative charge that repels the chloride ion and prevents its transport. Glyphosate substituting for this same glycine residue would also introduce a negative charge and disrupt chloride transport in the same way. Genetic mutations involving substitutions of arginine, glutamate or alanine for glycines at positions 319, 193, 243, and 478 in NKCC2 are all linked to antenatal Bartter Syndrome expression [109,110].

Conclusion

Glyphosate degrades human health in a number of ways, but its most pernicious action may be its substitution for glycine during protein synthesis. In a companion paper we show how glyphosate amplifies kidney injury from known risk factors such as NSAIDs and dehydration. Here we hypothesize that glyphosate's misincorporation during protein synthesis in place of glycine, the second most common amino acid in humans, causes a cascade of metabolic and homeostatic changes that result in catastrophic renal damage.

We have shown how multiple proteins critical for kidney health, particularly under heat stress and exercise stress, could be disrupted by glyphosate substitution for critical glycine residues within these proteins. In particular, defective aquaporin would enhance vulnerability to dehydration, and defective chloride channels would impair acidification of the lysosome, with multiple implications, particularly in the context of muscle damage and rhabdomyolysis. Impaired Na^+/H^+ exchangers would lead to increased acidification of urine, as is observed in MeN. It is remarkable how many crucial functions in the kidney would be disrupted by impaired megalin-based endocytosis, which could be induced by glyphosate misincorporation into glycine in multiple proteins involved with megalin processing. Table 3 summarizes various pathologies that can be predicted to arise from glyphosate exposure in the kidney, assuming that essential glycine residues are being displaced by glyphosate. It is likely that many other proteins with critical dependencies on glycine residues that we have not yet identified are also being adversely affected by glyphosate. The need for action is urgent. CKDu is a public health crisis that could be significantly addressed by discontinuing the use of glyphosate-based herbicides on sugarcane.

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