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The role of L-type amino acid transporters in the uptake of glyphosate across mammalian epithelial tissues

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HIGHLIGHTS

- Transport properties of glyphosate were investigated using Caco-2 and nasal mucosal tissues.
- Inhibitors were used to investigate the role of LAT transporters in glyphosate permeation in both tissues.
- Glyphosate permeation across both epithelia is mediated by amino acid transporters.

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ABSTRACT

Glyphosate is one of the most commonly used herbicides worldwide due to its broad spectrum of activity and reported low toxicity to humans. Glyphosate has an amino acid-like structure that is highly polar and shows low bioavailability following oral ingestion and low systemic toxicity following intravenous exposures. Spray applications of glyphosate in agricultural or residential settings can result in topical or inhalation exposures to the herbicide. Limited systemic exposure to glyphosate occurs following skin contact, and pulmonary exposure has also been reported to be low. The results of nasal inhalation exposures, however, have not been evaluated. To investigate the mechanisms of glyphosate absorption across epithelial tissues, the permeation of glyphosate across Caco-2 cells, a gastrointestinal epithelium model, was compared with permeation across nasal respiratory and olfactory tissues excised from cows. Saturable glyphosate uptake was seen in all three tissues, indicating the activity of epithelial transporters. The uptake was shown to be ATP and Na^+ independent, and glyphosate permeability could be significantly reduced by the inclusion of competitive amino acids or specific LAT1/LAT2 transporter inhibitors. The pattern of inhibition of glyphosate permeability across Caco-2 and nasal mucosal tissues suggests that LAT1/2 play major roles in the transport of this amino-acid-like herbicide. Enhanced uptake into the epithelial cells at barrier mucosae, including the respiratory and gastrointestinal tracts, may result in more significant local and systemic effects than predicted from glyphosate's passive permeability, and enhanced uptake by the olfactory mucosa may result in further CNS disposition, potentially increasing the risk for brain-related toxicities.

1. Introduction

Glyphosate [N-(phosphonomethyl) glycine], an herbicide which has been commercially available since 1974, is commonly used both in large agriculture settings and for residential purposes (Mink et al., 2011). Many commercial products containing glyphosate as the active ingredient have been used worldwide due to their effectiveness and relatively low toxicity to humans and other animals (Jasper et al., 2012). As an herbicide, glyphosate inhibits the 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) enzyme, which acts in the biosynthesis of aromatic amino acids and of shikimic acid. The enzyme is not present in mammals, which reduces the likelihood for any direct toxicity for glyphosate in

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humans (Kimmel et al., 2013; Prevot-D'Alvise et al., 2013).

Glyphosate is a broad-spectrum, non-selective, post-emergence herbicide, typically sprayed over the areas being treated. As a result, both inhalation and topical exposures to glyphosate are possible, both for the applicator and for other humans and animals in the area of spray application (Wester et al., 1991; Meza-Joya et al., 2013). Given the frequency of spray applications using glyphosate, nasal inhalation exposure to the spray is extremely likely to occur. While there are no current reports regarding potential glyphosate toxicities following nasal inhalation, studies in cell culture (Slaninova et al., 2009; Koller et al., 2012; Mesnage et al., 2013; Prevot-D'Alvise et al., 2013) have suggested potential toxicities to several human cell types, including those obtained from the buccal, nasal, and lower gastrointestinal tract, and other investigations have demonstrated toxicity to the olfactory systems of several species of fish (Slaninova et al., 2009; Solomon et al., 2013). Although glyphosate itself has been shown to be extremely safe in humans following dermal exposure, toxicities from commercial products have been reported to be induced by the excipients contained in the products, especially the surfactants used to improve the permeability of glyphosate across plant cell walls (Gasnier et al., 2009; Allen and Levy, 2013).

Several investigators have described glyphosate distribution in Sprague Dawley rats following a single oral dose and showed that glyphosate has a limited distribution (1.98%) into the brain (Brewster et al., 1990; Williams et al., 2000). Most of the ingested glyphosate remained associated with the small intestinal tissues, likely being sequestered in the surface epithelial cells. Additional reports, however, continue to suggest a link between glyphosate exposure and a variety of adverse health effects, including Parkinson's and Alzheimer's disease, and recently, even cancer (Kamel et al., 2007; Freire and Koifman, 2012; Allen and Levy, 2013; Guyton et al., 2015).

In plants, glyphosate is reported to be a substrate for several phosphate transport systems (Denis and Delrot, 1993). Since glyphosate is a glycine analog, it is possible that glyphosate may also be a substrate for the glycine-uptake pathways in mammalian cells. A family of transport proteins, glycine transporters (GLYT), which are specific for glycine transport, and the $b^{0,+}$, ASC, asc and L systems of the amino acid transporter family (Kanai and Endou, 2001; Verrey, 2003; Del Amo et al., 2008), are also able to transfer glycine.

Amino acid transporters, including y^+ LAT, $b^{0,+}$, LAT1, and LAT2, are facilitative transporters which act by using a unique mechanism that is described as an “in and out” transfer, where the transporter effluxes one amino acid out of the cell to exchange for another amino acid taken up into the cell at the same time. Given the affinity of several amino-acid-analog drug compounds (L-dopa, gabapentin) for the L system transporters (LAT1, LAT2), the affinity of glyphosate at these transporters was the focus of these investigations. The activity of other, non-LAT transporters was also considered in these studies since there are numerous reports of the overlapping substrate specificities among the amino acid transporters (Del Amo et al., 2008).

If amino acid transport systems are involved in the systemic absorption and distribution of glyphosate, or potentially act to allow the nose-to-brain transfer of glyphosate across the nasal epithelium, the potential for nasal inhalation toxicity may be greater than has been predicted from the previously described pulmonary toxicity profiles (Bradberry et al., 2004). In order to investigate the epithelial transfer properties of glyphosate, two different *in vitro* models were used to evaluate the roles of passive permeability and carrier-mediated uptake. Absorption across the gastrointestinal epithelium was measured using Caco-2 cells, a transformed colonic epithelial cell line frequently used to investigate the gastrointestinal permeability of drug compounds. Excised bovine nasal mucosal tissues were employed to evaluate the uptake of glyphosate by the nasal epithelium and to compare the transport capacities between the nasal respiratory and olfactory mucosae. Previous investigators have shown that LAT1 and LAT2 are expressed in Caco-2 cells, and confirmation of the expression of these transporters throughout the intestinal tract of animals and humans has been reported (Fraga et al., 2005; Terada et al., 2005). The expression of LAT1 and LAT2 gene transcripts in the nasal olfactory and respiratory mucosa of humans, rats and cattle was recently reported by Al Ghabeish et al. (2015) using DNA microarray analysis. Weak expression of LAT1 in cattle mucosal tissues was reported by Zhang (2009) using immunoblotting, and Ferreira and Donovan (2013) more recently reported the confirmation of the expression and localization of LAT2 in cattle nasal mucosa using both PCR and immunohistochemical analyses.

2. Materials and methods

2.1. Reagents

Glyphosate, gabapentin HCl, L-alanine, L-leucine, L-phenylalanine, 2-amino-2-norbornanecarboxylic acid (BCH), tetraethylammonium (TEA) and 2,4-dinitrophenol (2,4-DNP) were obtained from Sigma–Aldrich Chemical (St. Louis, MO, USA). HPLC-fluorescence derivatization agent FMOC-Cl and GC-FID derivatization agents, including trifluoroacetic anhydride (TFAA), trifluoroethanol (TFE) and chloroform were also obtained from Sigma–Aldrich Chemical. Dichloromethane and acetonitrile were purchased from Thermo Fisher Scientific (Waltham, MA, USA).

Krebs Ringer's buffer (KRB) was composed of 1.67 mM $MgCl_2$, 4.56 mM KCl, 119.78 mM NaCl, 1.5 mM NaH_2PO_4, 0.83 mM Na_2HPO_4, 10 mM D-glucose, and 15 mM $NaHCO_3$ in 1000 mL deionized water. After bubbling with 95% CO_2/5% O_2, 1.2 mM $CaCl_2$ was added. The pH was adjusted to 7.4 using 1 M HCl. TEA was used in the Na^+ free medium instead of NaCl, and other Na^+ containing compounds were substituted with K^+ salts.

Hank's balanced salt solution (HBSS) was composed of 137 mM NaCl, 5.4 mM KCl, 0.44 mM KH_2PO_4, 0.35 mM Na_2HPO_4, 5.55 mM D-glucose, 1.26 mM $CaCl_2$, 0.32 mM $MgCl_2$ and 4.2 mM $NaHCO_3$ in 1000 mL deionized water, and then filtered with a 0.22 μm filter for sterilization. For the Na^+ free HBSS, only NaCl was substituted with TEA (137 mM) while other Na^+ containing compounds were substituted with K^+ salts. All of the chemicals used to prepare the buffer were obtained from Sigma–Aldrich Chemical.

2.2. Glyphosate transport across nasal tissues

2.2.1. Preparation of nasal mucosal tissues

Bovine nasal mucosal tissues were obtained from Bud's Custom Meat (Riverside, IA, USA and Wufeng Meat Corporation, Wuhan, China). The nasal turbinates were retrieved by opening the nasal cavity along the septal midline and removing the turbinate from the lateral wall. The ethmoturbinate, which is covered by olfactory mucosa and the maxilloturbinates, which are covered by respiratory mucosa, were excised carefully and the tissues were immediately transported to the laboratory in ice-cold KRB.

The mucosal tissues were stripped from the underlying cartilage using forceps and returned to the ice-cold KRB prior to mounting onto Navicyte® (Harvard Apparatus, Holliston, MA, USA) vertical diffusion chambers. Once placed into the chambers, the tissues were equilibrated at 37 °C in KRB for 15 min. Carbogen was used to mix, oxygenate, and maintain the buffer pH by bubbling into the donor and receiver chambers at a rate of 3–4 bubbles per second.