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Phylogenetic origins for severe acetaminophen toxicity in snake species compared to other vertebrate taxa

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ABSTRACT

While it has been known for a while that some snake species are extremely sensitive to acetaminophen, the underlying mechanism for this toxicity has not been reported. To investigate if essential detoxification enzymes are missing in snake species that are responsible for biotransformation of acetaminophen in other vertebrate species, livers were collected from a variety of snake species, together with samples from alligator, snapping turtle, cat, rat, and cattle. Subcellular fractions were analyzed for enzymatic activities of phenol-type sulfotransferase and UDP-glucuronosyltransferase, total glutathione S-transferase, and *N*-acetyltransferase. The results showed that none of the snake species, together with the cat samples, had any phenol-type glucuronidation activity, and that this activity was much lower in alligator and turtle samples than in the mammalian species. Combined with the lack of *N*-acetyltransferase activity in snakes and cats, this would explain the accumulation of the aminophenol metabolite, which induces methemoglobinemia and subsequent suffocation of snakes and cats after acetaminophen exposure. While previous investigations have concluded that in cats the gene for the phenol-type glucuronosyltransferase isoform has turned into a pseudogene because of several point mutations, evaluation of genomic information for snake species revealed that they have only 2 genes that may code for glucuronosyltransferase isoforms. Similarity of these genes with mammalian genes is < 50%, and suggests that the expressed enzymes may act on other types of substrates than aromatic amines. This indicates that the extreme sensitivity for acetaminophen in snakes is based on a different phylogenetic origin than the sensitivity observed in cats.

1. Introduction

After the accidental introduction of the brown treesnake (*Boiga irregularis*) on the island of Guam in the 1950s, the population of this species rapidly expanded because of the lack of natural predators and the presence of a bountiful array of prey species (Savidge, 1987). After several decades of expansion, a number of indigenous bird species are now considered extinct on the island, and the brown treesnake is considered a nuisance species for human activities (Rodda et al., 1999; Burnett et al., 2012). This triggered investigations into possible methods to contain and eradicate this invasive species. A variety of general wildlife pesticides was tested for their toxicity to the brown treesnake, together with several human therapeutic drugs that were known to be toxic to some vertebrate species. Surprisingly, the brown treesnake proved to be very sensitive to low doses of acetaminophen, and to a lesser extent to aspirin, but not ibuprofen (Savarie et al., 2000). A dose of only 80 mg acetaminophen per animal did kill 100% of the tested snakes within 12–24 h. This knowledge has since been used to control the brown treesnake population on Guam by lacing dead mice with acetaminophen, and distributing this bait in habitats where the snakes reside. Brown treesnakes do eat dead carrion, and therefore this has proven to be an effective and relatively safe management strategy (Johnston et al., 2002).

From a comparative toxicology point of view it was unexpected that this species is so sensitive to acetaminophen. The compound is used as a common over-the-counter analgesic, and has relatively low toxicity to humans and most other mammals (Bertolini et al., 2006). After absorption and distribution, a large amount of the compound is processed in the liver, where specific isoforms of two enzymes, sulfotransferase (SULT) and UDP-glucuronosyltransferase (UGT), rapidly conjugate the acetaminophen with a sulfonate group or a sugar group, and thus ready the poorly water soluble substrate for excretion in urine or bile (Bertolini et al., 2006). If these enzymatic pathways become saturated, another liver enzyme (cytochrome P-450-2E1) can turn the

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acetaminophen into a highly reactive quinone (NAPQI), which can cause liver damage, as seen in people who overdose on the drug (James et al., 2003). The reactive quinone can be neutralized by the anti-oxidant glutathione, with help of the enzyme glutathione S-transferase. But if this pathway becomes saturated after an excessive dose, and accumulation of NAPQI occurs, serious liver damage will ensue (McGill and Jaeschke, 2013).

This well-studied pathology profile of acetaminophen in humans could explain the observed toxicity in the brown treesnake if snakes are missing any of the essential enzymes in the detoxification pathway of the compound. However, from experiments in which brown treesnakes were dosed with acetaminophen, it became clear that they did not die from acute liver failure, but from anemic hypoxia (Clark et al., 2018). This rare phenomenon is also seen in feline species, like cats, when they are exposed to acetaminophen (Court and Greenblatt, 1997). The sensitivity of cats to acetaminophen has been explained by the lack of a functional isoform of the UDP-glucuronosyltransferases which conjugates acetaminophen in other mammalian species. The gene for this isozyme has several point mutations in cats, which has turned the gene into a pseudo-gene (Court and Greenblatt, 2000). Because the lack of a functional phenol-type UGT isoform leads to acute toxicity in cats, the objective of the current study was to investigate if the underlying mechanism for acetaminophen toxicity in cats is the same as in the brown treesnake and other snake species, or if phylogenetic signals leading to differentiated enzyme expressions are responsible for the sensitivity of reptilians to acetaminophen. The approach to answer this question was to collect liver samples from a variety of snake species, and several other vertebrates for comparison, and measure the activities of the enzymes involved in acetaminophen metabolism. In addition, we explored existing data in GenBank of investigated or closely related species to compare genetic information on the genes involved in these enzymatic pathways.

2. Materials and methods

2.1. Tissue samples

Eastern diamondback rattlesnake (*Crotalus adamanteus*) samples (n = 2) were kindly donated by Darin Rokyta's lab (Florida State University, Tallahassee, FL), ball python (*Python regius*) and corn snake (*Pantherophis guttatus*) samples (both n = 1) were obtained from University of Texas Arlington (Todd Castoe lab). Liver samples (n = 1 each) of several Colubridae snakes (*Nerodia clarkii compressicauda*, *Phyllorhynchus decurtatus*, *Rhinocheilus lecontei*, *Thamnophis marcianus*) and a cottonmouth (*Agkistrodon piscivorus*) were donated by Chris Parkinson's lab (University of Central Florida, Orlando, FL). Samples of Burmese python (*Python bivittatus*, n = 4) were obtained with the help of Frank Mazzotti's lab (University of Florida, Davie, FL). American alligator (*Alligator mississippiensis*) samples (n = 5) were supplied by Lou Gillette's lab (Medical University of South Carolina, Charleston, SC). Snapping turtles (*Chelydra serpentine*, n = 4) were collected locally in the Reedy River near Greenville, SC, rat (*Rattus norvegicus*) samples (n = 4) were obtained from the Godley Snell animal use facility at Clemson University, cat (*Felis catus*) livers (n = 4) were dissected from euthanized feral cats at the Oconee Animal Shelter (Seneca, SC) and heifer (*Bos taurus*) livers (n = 6) were obtained from the veterinary school at the University of Georgia (Athens, GA). All liver tissue samples were flash frozen in liquid nitrogen and stored at −80 °C until use.

Livers were thawed on ice and approximately 2 g of liver tissue was homogenized with a Polytron tissue grinder in 10 ml ice-cold Tris buffer (0.05 M, pH 7.4), containing 0.25 M sucrose, 1 mM ethylenediaminetetraacetic acid (EDTA), 0.1 mM dithiothreitol (DTT) and 0.2 mM phenylmethanesulfonyl fluoride (PMSF). Smaller samples were homogenized with a glass Potter-Elvehjem homogenizer in 2 ml of chilled homogenization buffer. Samples were spun for 20 min at 10,000 g at 4 °C to remove cell debris, connective tissue and fat, followed by a 60 min cold spin at 100,000 g. The cytosolic supernatant was collected and separated in aliquots; the microsomal pellet was then resuspended in 1 ml Hepes buffer (0.01 M, pH 7.4), containing 0.25 M sucrose, 0.1 mM EDTA, 0.1 mM DTT, and 5% glycerol. Samples were frozen and stored at −80 °C until use.

2.2. Protein assay

All protein concentrations were measured with a bicinchoninic acid (BCA) Protein Assay Kit (Pierce, Rockford, IL), using bovine serum albumin (BSA) to prepare the standard curve.

2.3. UDP-glucuronosyltransferase activity

UGT activity was measured using 1-naphthol as a substrate (Mackenzie and Hänninen, 1980), which is a good substrate for the UGT isoform that is also responsible for the conjugation of acetaminophen. The benefit of using 1-naphthol is that this substrate and its metabolite are fluorescent, which lowers the detection limit in spectrophotometric analysis (Soikkeli et al., 2011). After method development experiments with varying concentrations of substrate, cofactor, microsomes, and Brij58, and different incubation times, the final assays were performed in a 250 µl reaction mixture, containing 0.1 M sodium phosphate buffer pH 7.4, 5 mM magnesium chloride, 25 µg of microsomal protein, treated with 0.3 mg/mg Brij 58, 0.08 mM 1-naphthol (20 µl from a 1 mM stock solution in 5% DMSO), and 0.1 mM uridine-diphosphoglucuronic acid (UDPGA). Negative controls consisted of the complete reaction mixture, but with a subsample of mixed microsomes that was boiled for 5 min to denature all proteins and thus inhibit any enzyme activity. The reaction was started by adding the UDPGA to the reaction mix. The reaction was performed at room temperature in an all-black 96-well microplate, with 3 replicate wells per sample. The production of the glucuronidated conjugate of 1-naphthol was measured at 293/335 nm (excitation/emission) over 30 min at 2 min intervals, using the kinetic option in SoftMax Pro software with a SpectraMax Gemini plate reader from Molecular Devices. The results are presented as V_{max} values (mUnits/min).

2.4. Sulfotransferase activity

Phenol-type sulfotransferase activity was measured based on the method published by Arand et al. (1987). After initial experiments to optimize substrate, cytosol, and cofactor concentrations, and incubation times, the assay mixture for the reported results consisted of 80 µl of a 1 M potassium phosphate buffer, pH 7.4, 200 µl of cytosol, adjusted to 1 mg/ml protein, and 100 µl of 3′-phosphoadenosine-5′-phosphosulfate (PAPS) of 0.1 mM, which was purified by retaining it on a Sep Pak Accell Plus QMA column (Waters Corporation, Milford, MA), and eluted with 150 mM sodium chloride. Negative controls consisted of the complete reaction mixture, but a subsample of mixed cytosol was boiled for 5 min to denature all proteins and thus inhibit any enzyme activity. The reaction was started by adding 20 µl of 1 mM β-naphthol in 5% dimethyl sulfoxide. The reaction mixture was incubated for 10 min at 37 °C, after which the reaction was stopped by adding 600 µl of 0.4 M glycine solution, acidified with 10% trichloroacetic acid to pH 2.2. The reaction products were separated by adding 5 ml of chloroform, vortexing for 30 s and centrifuging at 2000*g* for 5 min to separate the phases. One hundred microliters of the upper phase was mixed with 140 µl of 1 N sodium hydroxide in all-black 96-microwell plates, and fluorescence was measured at 285/335 nm excitation/emission wavelengths in a Biotek plate reader. Reaction rates were calculated using a 0.625–10 mM standard curve of 2-naphthyl sulfate potassium salt.