Available online at www.sciencedirect.com
ScienceDirect
Comparative Biochemistry and Physiology, Part A 145 (2006) 419–422
CBP
www.elsevier.com/locate/cbpa

Detoxification rates of wild herbivorous woodrats (*Neotoma*)

M.D. Dearing *, M.M. Skopec, M.J. Bastiani

Biology Department, 257 S 1400 E, University of Utah, Salt Lake City, UT 84112, USA

Received 28 April 2006; received in revised form 26 July 2006; accepted 29 July 2006
Available online 4 August 2006

Abstract

The detoxification systems of mammalian herbivores are thought to have evolved in response to the ingestion of plant secondary compounds. Specialist herbivores consume high quantities of secondary compounds and are predicted to have faster rates of Phase 1 detoxification compared to generalist herbivores. We tested this hypothesis by comparing the performances of a specialist (*Neotoma fuscipes*) and generalist (*Neotoma lepida*) herbivore using hypnotic state assays. Herbivores foraging in nature were live trapped and injected with hexobarbital (100 mg/kg). We measured the length of time in the hypnotic state as the time in which the animal was unable to right itself twice in 30 s. The specialist metabolized hexobarbital 1.7 times faster than the generalist ($F_{1,\ 19}=9.31$, $P=0.007$) as revealed by its significantly shorter time spent in the hypnotic state (56±9 min vs. 87±8 min, respectively). The results are consistent with the hypothesis that specialists have faster rates of Phase 1 detoxification. This is the first evaluation of the detoxification capability of mammalian herbivores foraging under natural conditions. Hypnotic state assays have broad potential applications to the study of vertebrate-plant interactions.

Keywords: Detoxification; Herbivores; Hexobarbital; Hypnotic state assay; *Neotoma*; Sleep time; Woodrat

1. Introduction

The detoxification systems of mammalian herbivores are thought to have evolved in response to the concentration and diversity of plant secondary compounds (PSCs) in their diet (Freeland and Janzen, 1974). Specialist herbivores tend to consume diets rich in PSCs and are predicted to possess detoxification systems that can metabolize large concentrations of chemically similar toxins while simultaneously minimizing energetic costs (Freeland and Janzen, 1974; Lamb et al., 2004). In particular, specialists are predicted to have high capacities of Phase 1 detoxification enzymes such as the cytochrome P450s (Boyle et al., 2000, 2001; Lamb et al., 2004). In contrast, generalist herbivores consume small amounts of various PSCs and are expected to utilize Phase 2 conjugation enzymes to process the diversity of ingested compounds (Boyle et al., 2000, 2001; Lamb et al., 2004). Phase 2 detoxification enzymes are less substrate specific than Phase 1 enzymes but require energetically costly conjugates such as glucose derivatives (Klaassen, 1996).

This long-standing detoxification hypothesis has been difficult to address for a number of reasons. First, although a number of *in vitro* techniques (e.g., microsomal assays) are commonly used in pharmacological studies of laboratory rats, such techniques have only recently been applied to mammalian herbivores (Pass et al., 2001, 2002). A limitation of many *in vitro* pharmacological approaches is that as endpoint studies, they can only be conducted on species for which adequate numbers can be harvested (8–10 individuals per treatment). Thus, they are not appropriate for endangered or protected species. Second, application of *in vitro* approaches is typically conducted on captive populations of herbivores to permit control of dietary contents and measurement of intake. However, such studies necessitate the use of artificial diets, which can introduce artifacts (Price et al., 1980, 2004; Appel, 1993).

We used a hypnotic state assay to test the detoxification hypothesis in free-ranging mammalian herbivores. A benefit of these assays is that they are relatively non-invasive, requiring only intraperitoneal injections of anesthesia from which the animals recover quickly (within a few hours). Hypnotic state assays are commonly used in nutraceutical or pharmacological studies to screen for possible drug interactions (Price et al., 2004; Kim and Shin, 2005; Nyarko et al., 2005; Oliveira et al.,

* Corresponding author.
E-mail address: dearing@biology.utah.edu (M.D. Dearing).

1095-6433/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.cbpa.2006.07.016

2005). The basic premise of the hypnotic state assay is that an animal is given a hypnotic agent (e.g., hexobarbital, zoxazolamine) and the length of time the animal is in a hypnotic state is measured (Kim et al., 1993; Sasaki, 1994). The length of time in the hypnotic state is inversely proportional to the animal's ability to metabolize the hypnotic agent. We selected hexobarbital as the hypnotic agent for this study as an indicator of Phase 1 detoxification.

The mammalian herbivores used in this study were a sympatric specialist and generalist. *Neotoma fuscipes* is a specialist on oak foliage (e.g., *Quercus agrifolia*). In parts of California, the specialist occurs sympatrically with another woodrat, *Neotoma lepida*, which has greater diet diversity. The diet of the *N. lepida* in this part of its range is dominated by *Opuntia occidentalis* cactus and *Salvia apiana* (Atsatt and Ingram, 1983). We tested the hypothesis that the specialist has greater rates of Phase 1 detoxification than the generalist by injecting woodrats with hexobarbital and measuring the length of time in the hypnotic state. If the specialist has faster rates of Phase 1 detoxification, we predicted it should metabolize hexobarbital faster resulting in shorter hypnotic state times compared to the generalist woodrat.

2. Methods

Woodrats *N. fuscipes* and *N. lepida* were trapped with Sherman live traps (30×9×8 cm) at Caspers Wilderness Park, San Juan Capistrano, CA in mid June 2005. The habitat consisted of oak forest edges abutting areas of cactus (*Opuntia*) flats. Traps were set at woodrat houses ("middens") and were opened at dusk and collected the following morning at sunrise. Woodrats were identified to species using morphological characters (male genitalia) and weighed to the nearest 0.1 g with a portable balance (Mettler ED601). We restricted our study to adult male woodrats (>130 g) because females could have been pregnant or nursing at the time of capture, and such reproductive states could affect the results. We captured 8 male *N. fuscipes* and 12 male *N. lepida* for use in the study.

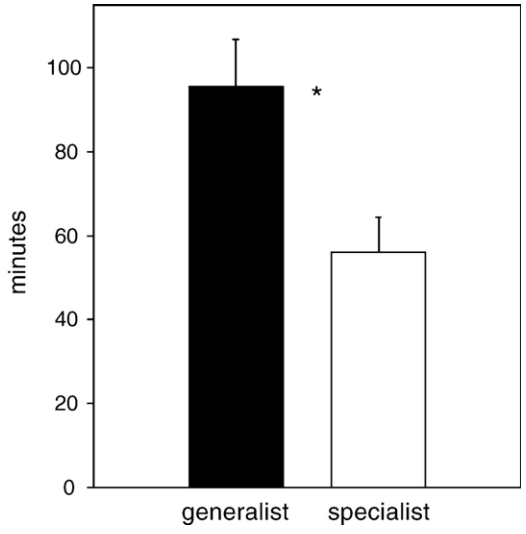

Fig. 1. Length of time in the hypnotic state for a generalist woodrat, *N. lepida* (solid bar) and a specialist woodrat, *N. fuscipes* (open bar). Asterisk indicates a significant difference ($P=0.007$) between the two species.

Male woodrats (were given an intraperitoneal injection of 100 mg/kg of hexobarbital. Hexobarbital was dissolved in 0.9% saline 0.25 N NaOH (50 mg/mL). Injection volumes ranged from 0.23 to 0.38 mL. After injection, woodrats were placed in a standard laboratory rat cage (32×23×19 cm). We measured "sleep time" beginning at the time woodrats lost the ability to right themselves twice within 30 s after being placed on their backs to the time when they could right themselves twice within 30 s after being placed on their backs (Koizumi et al., 2002). Animals were handled according to IACUC protocol 04-0212.

Sleep times were not normally distributed and were $\log(x+1)$ transformed for analysis. We compared sleep times between the specialist and generalist using a one-way ANOVA with species as the independent factor. We investigated the possible relationship between body size and sleep time independently for each species with linear regression. All analyses were conducted in JMP 4.0.4.

3. Results

Male *N. fuscipes* ($x=176\pm3.4$ g) were 5.9% larger than the male *N. lepida* ($x=166\pm2.8$; $F_{1,19}=5.5$, $P=0.03$).

Sleep times differed significantly between the specialist and the generalist ($F_{1,19}=9.31$, $P=0.007$). The generalist slept 1.7 times longer than the specialist (Fig. 1). There was no relationship between sleep time and body size for either the specialist ($r^2=0.07$, $P=0.51$) or the generalist ($r^2=0.07$, $P=0.43$).

4. Discussion

We tested the hypothesis that specialist herbivores have faster rates of Phase 1 detoxification than generalists (Boyle, 2000; Boyle et al., 2001; Lamb et al., 2004). The data from this study are consistent with this hypothesis in that the specialist herbivore metabolized hexobarbital almost twice as fast as the generalist herbivore. Future studies on more specialist and generalist herbivores are needed to determine whether this hypothesis is applicable to herbivores in general. To our knowledge, these data are the first on detoxification rates of mammalian herbivores consuming natural diets under field conditions. In the subsequent paragraphs, we discuss the implications of these results with respect to dietary strategy, detoxification physiology, putative secondary compounds, and genetic basis. We also present ideas on how this assay could be applied to other studies.

The detoxification systems of mammals consist of more than 100 enzymes (Klaasen, 1996). Across mammals, much conservation exists in these enzymes, although species-specific differences are not uncommon. With respect to woodrats, the molecular details (e.g., DNA sequences, molecular structures) have not been characterized for each enzyme in the detoxification system. However, data from model systems can be used to infer the enzymes in woodrats responsible for metabolism of hexobarbital. Cytochrome P450s, specifically cytochrome 2B

(CYP 2B) is the most probable candidate for hexobarbital metabolism in woodrats. CYP 2B is the key enzyme in the metabolism of hexobarbital and other barbiturates by laboratory rats, *Rattus norvegicus* (Jori et al., 1970; Waxman and Azaroff, 1992; Lewis and Lake, 1997). Laboratory rats and woodrats are reasonably closely related in that they are in the same family of rodents (Muridae). More distantly related species such as rabbits and humans (Lewis and Lake, 1997) also primarily use CYP 2B to oxidize hexobarbital. Given the conservation in function across these broad taxonomic groups (rats, humans, rabbits), it is likely that CYP 2B is also responsible for hexobarbital metabolism in woodrats.

Were the observed differences in hexobarbital metabolism between the specialist and generalist the result of evolutionary (genetic) or ecological (diet) differences? The activity of a number of detoxification enzymes can be induced several fold through repeated exposure to xenobiotics such as ingested PSCs. The majority of the diet of the specialist consists of oak leaves, which contain high concentrations of phenolics such as quercetin (Mauffette and Oechel, 1989; Chauhan et al., 2004; Sakar et al., 2005). Quercetin and other phenolics compounds are metabolized by CYP 2B in laboratory rats (Debersac et al., 2001; Rahden-Staron et al., 2001). In contrast, the generalist at this location is not reported to feed on plants containing high levels of phenolics. Thus, it is possible that the enhanced ability of the specialist to metabolize hexobarbital is purely ecological and due to its greater consumption of PSC metabolized by CYP 2B compared to the generalist.

In contrast, studies in mice and humans suggest that differences in metabolism of barbital containing compounds are strongly influenced by genetic differences in detoxification enzymes and that genetics may trump environmental exposure (Knodell et al., 1988; Arthur et al., 2003). Large genetic differences in hexobarbital metabolism exist across human populations. Humans that are slow metabolizers of hexobarbital have plasma levels of hexobarbital that are 5× greater than fast metabolizers (Knodell et al., 1988). A recent study in mice (Arthur et al., 2003) revealed that genetics had a greater effect on metabolism of pentobarbital (chemically similar to hexobarbital) than dietary endophyte toxins. They found that in two lines of laboratory mice selected for either resistance or susceptibility to endophyte toxin, the resistant line metabolized pentobarbital 20% faster than the susceptible lines. Exposure to dietary endophyte toxin did not affect pentobarbital metabolism of either line compared to the control diet; i.e., the resistant lines metabolized pentobarbital 20% faster regardless of diet treatment. In our study, the magnitude of the difference in hexobarbital metabolism between woodrat species (specialist 70% faster than generalist) is consistent with underlying genetic differences. Further research is necessary to unequivocally distinguish the roles of evolution and ecology in this system.

There is tremendous potential for use of hypnotic state assays in understanding detoxification of PSC by wild herbivores (Sorensen et al., 2006). This technique is reasonably noninvasive and requires little animal time in captivity. Thus, it could be applied to species of special concern (endangered, listed) where terminal studies are not possible. Hypnotic state assays may facilitate investigations of animals difficult to work with in captivity either because they do not accept artificial diets (red voles, koalas) or because they do not respond well to captivity (pikas, rabbits). In addition, other hypnotic agents can be used to probe the capacities of other detoxification enzymes. For example, zoxazolamine is primarily metabolized by CYP 1A (Jori et al., 1970; Atal et al., 1985).

Lastly, if animals can be kept in captivity for 3–4 days, it would be possible to distinguish between the roles of genetic and ecological factors in detoxification. In this approach, animals would be treated with a hypnotic agent immediately after capture as conducted in this study. Subsequent doses of the hypnotic agent would be administered daily over the next three days to fully induce the detoxification enzymes under study. The length of the hypnotic state on day 3 should be representative of maximally induced states. At that point, any differences among the treatment groups are likely the result of underlying genetic differences given that they have had similar induction exposure to the hypnotic agent. It would be possible to measure constitutive enzyme levels by conducting a reciprocal study of maintaining animals on a toxin free diet and then measuring length of hypnotic state. However, this study would require at least a week in captivity on a toxin-free diet as the down regulation of detoxification enzymes may be longer than induction times (Pass et al., 2001).

Acknowledgements

NSF (IBN 0236402) provided the funding for this research. We thank Cheryl Votaw for preparation of field equipment, Kathy Smith for manuscript preparation and Casper's Wilderness Park for access to the study areas. Two anonymous reviewers provided helpful comments.

References

Appel, H.M., 1993. Phenolics in ecological interactions: The importance of oxidation. J. Chem. Ecol. 19, 1521–1552.

Arthur, K.A., Kuehn, L.A., Hohenboken, W.D., 2003. Sleep time following anesthesia in mouse lines selected for resistance or susceptibility to fescue toxicosis. J. Anim. Sci. 81, 2562–2567.

Atal, C.K., Dubey, R.K., Singh, J., 1985. Biochemical basis of enhanced drug bioavailability by piperine evidence that piperine is a potent inhibitor of drug metabolism. J. Pharmacol. Exp. Ther. 232, 258–262.

Atsatt, P.R., Ingram, T., 1983. Adaptation to oak and other fibrous phenolic-rich foliage by a small mammal *Neotoma fuscipes*. Oecologia (Berlin) 60, 135–142.

Boyle, R., 2000. Metabolic fate of dietary terpenes in folivorous marsupials. Tasmanian School of Pharmacy. University of Tasmania, Hobart, p. 217.

Boyle, R., McLean, S., Foley, W.J., Moore, B.D., Davies, N.W., Brandon, S., 2000. Fate of the dietary terpene, *p*-cymene, in the male koala. J. Chem. Ecol. 26, 1095–1111.

Boyle, R., McLean, S., Foley, W., Davies, N.W., Peacock, E.J., Moore, B., 2001. Metabolites of dietary 1,8-cineole in the male koala (*Phascolarctos cinereus*). Comp. Biochem. Physiol. C, Comp. Pharmacol. Toxicol. 129, 385–395.

Chauhan, S.M.S., Singh, M., Kumar, A., 2004. Isolation and characterization of selected secondary metabolites from dry leaves of *Quercus semicarpifolia*. Indian J. Chem., Sect. B 43, 223–226.

Debersac, P., Heydel, J.M., Amiot, M.J., Goudonnet, H., Artur, Y., Suschetet, M., Siess, M.H., 2001. Induction of cytochrome P450 and/or detoxication enzymes by various extracts of rosemary: description of specific patterns. Food Chem. Toxicol. 39, 907–918.

Freeland, W.J., Janzen, D.H., 1974. Strategies in herbivory by mammals: The role of plant secondary compounds. Am. Nat. 108, 269–289.

Jori, A., Bianchetti, A., Prestini, P.E., 1970. Relations between barbiturate brain levels and sleeping time in various experimental conditions. Biochem. Pharmacol. 19, 2687–2694.

Kim, E.J., Shin, W.H., 2005. General pharmacology of CKD-732, a new anticancer agent: Effects on central nervous, cardiovascular, and respiratory system. Biol. Pharm. Bull. 28, 217–223.

Kim, N.D., Yoo, J.K., Won, S.M., Park, S.S., Gelboin, H.V., 1993. Phenytoin induction of cytochrome P4502B in mice: Effects on hexobarbital hydroxylase activity. Xenobiotica 23, 217–225.

Klaasen, C.D., 1996. Casarett and Doull's Toxicology: the Basic Science of Poisons. McGraw-Hill.

Koizumi, T., Maeda, H., Hioki, K., 2002. Sleep-time variation for ethanol and the hypnotic drugs tribromoethanol, urethane, pentobarbital, and propofol within outbred ICR mice. Exp. Anim. (Tokyo) 51, 119–124.

Lamb, J.G., Marick, P., Sorensen, J., Haley, S., Dearing, M.D., 2004. Liver biotransforming enzymes in woodrats *Neotoma stepensi* (Muridae). Comp. Biochem. Physiol. C, Comp. Pharmacol. Toxicol. 138, 195–201.

Lewis, D.F.V., Lake, B.G., 1997. Molecular modelling of mammalian CYP2B isoforms and their interaction with substrates, inhibitors and redox partners. Xenobiotica 27, 443–478.

Mauffette, Y., Oechel, W.C., 1989. Seasonal variation in leaf chemistry of the coast live oak *Quercus agrifolia* and implications for the California oak moth *Phryganidia californica*. Oecologia (Berlin) 79, 439–445.

Nyarko, A.K., Okine, L.K., Wedzi, R.K., Addo, P.A., Ofosuhene, M., 2005. Subchronic toxicity studies of the antidiabetic herbal preparation ADD-199 in the rat: absence of organ toxicity and modulation of cytochrome P450. J. Ethnopharmacol. 97, 319–325.

Oliveira, F.A., Costa, C.L.S., Chaves, M.H., Almeida, F.R.C., Cavalcante, I.J.M., Lima, A.F., Lima, R.C.P., Silva, R.M., Campos, A.R., Santos, F.A., Rao, V.S.N., 2005. Attenuation of capsaicin-induced acute and visceral nociceptive pain by alpha-and beta-amyrin, a triterpene mixture isolated from *Protium heptaphyllum* resin in mice. Life Sci. 77, 2942–2952.

Pass, G.J., McLean, S., Stupans, I., Davies, N., 2001. Microsomal metabolism of the terpene 1,8-cineole in the common brushtail possum (*Trichosurus vulpecula*), koala (*Phascolarctos cinereus*), rat and human. Xenobiotica 31, 205–221.

Pass, G.J., McLean, S., Stupans, I., Davies, N.W., 2002. Microsomal metabolism and enzyme kinetics of the terpene p-cymene in the common brushtail possum (*Trichosurus vulpecula*), koala (*Phascolarctos cinereus*) and rat. Xenobiotica 32, 383–397.

Price, M.L., Hagerman, A.E., Butler, L.G., 1980. Tannin in sorghum grain effect of cooking on chemical assays and on anti nutritional properties in rats. Nutr. Rep. Int. 21, 761–768.

Price, R.J., Scott, M.P., Walters, D.G., Stierum, R.H., Groten, J.P., Meredith, C., Lake, B.G., 2004. Effect of thiabendazole on some rat hepatic xenoblotic metabolising enzymes. Food Chem. Toxicol. 42, 899–908.

Rahden-Staron, I., Czeczot, H., Szumilo, M., 2001. Induction of rat liver cytochrome P450 isoenzymes CYP 1A and CYP 2B by different fungicides, nitrofurans, and quercetin. Mutat. Res. 498, 57–66.

Sakar, M.K., Sohretoglu, D., Ozalp, M., Ekizoglu, M., Piacente, S., Pizza, C., 2005. Polyphenolic compounds and antimicrobial activity of *Quercus aucheri* leaves. Turk. J. Chem. 29, 555–559.

Sasaki, N., 1994. Effects of furazolidone on duration of righting reflex loss induced with hexobarbital and zoxazolamine in the rat. J. Vet. Med. Sci. 56, 667–670.

Sorensen, J.S., Skopec, M.M., Dearing, M.D., 2006. Application of pharmacological approaches to plant–mammal interactions. J. Chem. Ecol. 32, 1229–1246.

Waxman, D.J., Azaroff, L., 1992. Phenobarbital induction of cytochrome p-450 gene expression. Biochem. J. 281, 577–592.