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A new method to measure intestinal activity of P-glycoprotein in avian and mammalian species

Accepted: 19 October 2004/Published online: 25 November 2004 © Springer-Verlag 2004

Abstract Permeability-glycoprotein (Pgp) actively exports numerous potentially toxic compounds once they diffuse into the cell membrane of intestinal epithelial cells. We adapted the everted sleeve technique to make the first measures of intestinal Pgp function in an avian species (chicken) and in wild mammalian species, and compared them to laboratory rats. Tissues maintained both structural and functional integrity, and our method offers advantages over other in vitro techniques by using smaller intestinal sections (1 cm), and shorter incubation times (8–12 min). To determine Pgp function, we compared accumulation of [3H]-digoxin in sleeves incubated in Ringer solution with and without a transport-saturating concentration of a competitive inhibitor, cyclosporin A. We demonstrated significant variation in Pgp activity within individuals along the intestine, between populations fed different diets, and between species (laboratory rats had one-third to one-fifth the Pgp activity of wild rodents). In chicken, we also tested the effect of natural metabolites on digoxin accumulation. We found that among flavonoids, genistein (200 μM), found in soy and other legumes, but not quercetin (10, 30, 100, 330 μM) or the 3-β-glycoside isoquercetin (100 μM), significantly increased digoxin accumulation. Among fungal metabolites, sterigmatocystin (5 μM), but not aflatoxin B1 (5 μM), significantly increased digoxin accumulation.

Keywords P-glycoprotein · Everted sleeve · Flavonoids · Aflatoxin · Sterigmatocystin

Abbreviations ABC: ATP-binding cassette · AFB1: Aflatoxin-B1 · CsA: Cyclosporin A · C.V.: Coefficient of variation · DMSO: Dimethyl sulfoxide · GEN: Genistein · ISOQ: Isoquercetin · MDR: Multi-drug resistance · PEG: Poly-ethylene glycol · Pgp: Permeability-glycoprotein · QUERC: Quercetin · SGLT-1: Sodium-dependent glucose transporter · ST: Sterigmatocystin

Introduction

This paper introduces a new application of a widely used technique for studying the regulatory and comparative physiology of intestinal epithelial transport. Our goal was to develop a method for measuring activity of the intestinal transporter Permeability-glycoprotein (Pgp) that could be applied to intestines of a wide variety of vertebrates and would provide the basis for comparisons of Pgp activity among species, populations or treatments within species. We adapted the everted sleeve method (Karasov and Diamond 1983), which offers advantages over other Pgp activity assays such as chamber techniques (Makhey et al. 1998; Stephens et al. 2001) that involve more complicated equipment and more difficult tissue preparation and mounting, and everted sac techniques (Barthe et al. 1998) that do not control for effects of the unstirred layer and require longer intestinal segments and increased incubation times.

Permeability-glycoprotein is a member of the ATP-binding cassette (ABC) superfamily that actively pumps out numerous potentially toxic endogenous and exogenous compounds once they diffuse into the cell membrane (Ambudkar et al. 1999, 2003). Pgp was first discovered when overexpression of this 170-kDa protein was correlated with cross-resistance to chemo-
therapeutics in cancer cells (Juliano and Ling 1976). Pgp has since been implicated as an important factor in the ability of cells exposed to a single drug/xenobiotic to develop resistance to a broad range of structurally and functionally unrelated drugs/xenobiotics, which has been termed multi-drug resistance (MDR) or multi-xenobiotic resistance (MXR) (Kurelec 1992; Hunter and Hirst 1997). Not limited to cancer cells, Pgp has been found on the secretory side of normal cells associated with secretion, absorption, or barrier function such as blood–brain barrier, blood–testis barrier, placenta, small intestine, pancreas, colon, adrenal cortex, kidney, and liver in humans and rodents (Hunter and Hirst 1997; Ambudkar et al. 1999) and gills and eggs in aquatic species (Bard 2000). However, little has been done to explore occurrence and function in other species. Our method will facilitate this exploration.

We were also eager to develop a simple method to test for the effects of synthetic and natural xenobiotics on intestinal Pgp activity. The ever-increasing list of substrates and modulators of Pgp includes a broad range of compounds from natural products such as anthracyclines, Vinca alkaloids, epipodophyllotoxins, colchicines, actinomycin D, and flavanoids, to pesticides and other pollutants (Ford and Hait 1990; Hunter and Hirst 1997; Ambudkar et al. 1999; Bard 2000). The only common characteristics appear to be that all are hydrophobic with a molecular mass between 300 and 2,000 Da (Ford and Hait 1990; Ambudkar et al. 2003). The tissue and cellular location of Pgp, ideal for mediation of toxin absorption, combined with the broad substrate recognition led to the hypothesis that Pgp plays a role in facilitating excretion and/or protection against the ubiquitous toxins found in diets and environments (Hunter and Hirst 1997; Epel 1998; Ambudkar et al. 1999). Numerous mammalian and avian species are continually exposed to both natural toxins in leaves, fruits, seeds, and insects in their diets, and human-made toxins as contaminants (herbicides, pesticides). However, there are few studies of Pgp in birds (Edelmann et al. 1999) and none addressing its potential role in the ecology of wild avian or mammalian species. Regarding the evolutionary physiology of Pgp, we hypothesize that species exposed to low levels of toxins in their natural diets exhibit relatively low levels of Pgp activity, similar to the patterns found with liver biotransformation enzymes (Walker 1980; Van Straalen 1994).

In this report, we describe experimental procedures and then validation experiments to select appropriate tissue incubation times and conditions. In order to illustrate the method’s power to detect differences among species, or among treatments within species, we report measures for several different mammalian and avian species and effects of different putative modulators in one species. Data are also presented on the ability of diet to alter Pgp activity. Materials and methods

Chemicals

[3H]-digoxin and [14C]-polyethylene glycol (PEG) were purchased from PerkinElmer Life and Analytical Sciences (Boston, MA, USA). [14C]-D-glucose and [3H]-L-glucose were purchased from Moravek (Brea, CA, USA). Quercetin, phloridzin, aflatoxin B1, and sterigmatocystin were purchased from Sigma Aldrich (St Louis, MO, USA). Isoquercetin and genistein were from Indofine (Hillsborough, NJ, USA). Cyclosporin A was purchased from Qbiogene, Inc. (Carlsbad, CA, USA).

Animals

Broiler-type chickens were purchased from Sunnyside farms (Beaver Dam, WI, USA), and Rhode Island Red Leghorn crosses (R×W) from the University of Wisconsin Poultry Research Laboratory. All birds were housed in heated batteries, at the poultry research laboratory on the University of Wisconsin-Madison campus, where they were fed a corn-soy based diet and had access to food and water ad libitum. Experiments were conducted on chickens 1–4 weeks old. Sprague-Dawley rats were purchased from Harlan Sprague-Dawley (Madison, WI, USA) and housed in temperature-controlled facilities in the Department of Wildlife Ecology, University of Wisconsin-Madison, with access to water and food ad libitum. All research conformed to approved UW-Madison Institutional Animal Use and Care Committee protocols.

Measures of Pgp activity using tissue accumulation of digoxin

For tissue preparation and mounting, we followed closely the procedures described in Karasov and Diamond (1983). Briefly, animals were euthanized, and the intestine from the distal end of the duodenal loop to the proximal end of the caecal attachment was quickly removed (<2 min) and flushed with ice-cold Ringer solution. Solution composition (in mM) was 50 or 100 mannitol (for mammals or birds, respectively), 100 NaCl, 4.7 KCl, 2.5 CaCl2, 1.2 KH2PO4, 1.2 MgSO4, and 20 NaHCO3, gassed with 95% O2 and 5% CO2. Osmolarity (mOsm) was 290 for mammals and 350 for birds. The intestine was everted, and short sleeves (1.5-cm-long) were cut and then mounted on stainless steel rods by tying the tissue down over grooves, exactly 1 cm apart. During this preparatory stage, mounted and unmounted tissues were kept in cold Ringer solution, gassed with 95% O2 and 5% CO2.

Just prior to a flux measurement, a rod with a mounted tissue was pre-incubated in 40°C (avian) or
37°C (mammal) Ringer solution, bubbled with 95% O2 and 5% CO2 for 2 min (Pgp assays) or up to 10 min (D-glucose uptake). Pre-incubation solutions also contained either test compounds or vehicle control to maximize tissue exposure to our treatments. The tissue was then transferred to a flat-bottomed water-jacketed test tube containing 8-ml-gassed Ringer solution with radiolabeled probe and marker solutes at 40°C (avian) or 37°C (mammal). The tube contained a stir bar spinning rapidly (approx. 1,000 rpm) to control unstirred layer thickness (Karsov and Diamond 1983). Probe molecules used were [3H]-digoxin (9.1 kBq/ml) for measuring Pgp activity (Sababi et al. 2001; Stephens et al. 2001), or [14C]-D-glucose (1.1 kBq/ml) for measuring D-glucose uptake activity. To correct for non-absorbed probe in adherent mucosal fluid, we used tracer concentrations of a membrane-impermeable marker [14C]-polyethylene glycol (PEG; MW = 4,000; 1.1 kBq/ml), or in the case of [14C]-D-glucose uptake we used [3H]-L-glucose (314.4 kBq/ml) to correct for both non-absorbed probe in adherent mucosal fluid and diffusive flux (Karsov and Diamond 1983). After a maximum of 15 min the incubation was terminated. Tissues were then either simply blotted (digoxin uptake) or rinsed in stirred, cold Ringer solution and then blotted (glucose transport). The mounted tissue was cut from between the two grooves with a razor blade, weighed, incubated in 1–1.75 ml of a tissue solubilizer (Soluene-350, Packard, Meriden, Conn.), and counted in a scintillation cocktail (10× volume of solubilizer; Ecolume, Packard) with 0.5% by volume acetic acid on a Wallac WinSpectral 1414 liquid scintillation counter (PerkinElmer Wallac Inc.), which corrects for variable quench and spill (counts of the alternate isotope appearing in the same counting channel). Calculation of digoxin accumulation followed Karsov and Diamond (1983), and for simplicity of expression we assumed a digoxin concentration of 1 fmol per µl incubation solution (it was actually approximately 0.75 fmol/µl), yielding units of fmol digoxin accumulated per milligram tissue.

Three kinds of trials were performed to optimize the method and evaluate its power for detecting differences within individuals, and within and among species.

**Cyclosporin A dose response**

First, we measured [3H]-digoxin uptake in the absence and presence of increasing concentration of cyclosporin A (1, 3.3, 10 µM, and saturated ~15 µM CsA), a known competitive inhibitor of Pgp (Lan et al. 1996). This was done because digoxin accumulation in the tissue is a balance between passive diffusion into and Pgp-mediated export out of the cells, so the desired measure of Pgp activity was the difference between digoxin accumulation when Pgp is maximally inhibited by the competitor and the accumulation in the absence of the competitor (Sababi et al. 2001). In these trials we used adjacent sleeves of intestine from the mid-intestinal regions, and the pre-incubation was 2 min in control (0.15% ethanol) or CsA-containing Ringer solution and the incubation was 8 min (avian) or 8 or 12 min (mammalian) in the same solutions with [3H]-digoxin. Measurements such as these were used to calculate an index of Pgp activity, defined as the difference in digoxin accumulation per mg tissue between tissues exposed to a maximal inhibitory concentration of CsA and to vehicle control (ethanol).

**Determination of incubation time**

Second, to determine optima, we progressively increased incubation time from 4 to 15 min and inspected the samples histologically (Starck et al. 2000) for villus damage, and calculated digoxin accumulation and our index for Pgp activity per unit time. In all the species tested, the shortest incubation time (4 min) was long enough to permit complete equilibration of adherent fluid with bathing solution (Karsov and Diamond 1983; Karsov et al. 1985), based on plateau values of PEG associated with tissue. In the time trials, we used adjacent sleeves of intestine incubated in solutions containing either a saturating concentration of CsA or no CsA. Pre-incubation time was consistently 2 min, making the total time that the tissue was exposed to heated Ringer solution 6–17 min.

**Determination of tissue viability and damage**

Third, as checks on the structural and functional integrity of the intestinal sleeves, we routinely inspected adjacent tissues histologically for signs of villus damage (Starck et al. 2000), and we measured mediated D-glucose uptake in both chicken and rat over the same combined pre-incubation and incubation time, using adjacent tissues exposed to CsA (10 µM), vehicle control (1% ethanol), and phloridzin (1 mM), a known inhibitor of the intestinal glucose transporter, SGTL-1. We also measured mediated D-glucose uptake using the putative inhibitors found to significantly affect [3H]-digoxin accumulation and the corresponding vehicle control. Finally, because we sometimes used ethanol and dimethyl sulfoxide (DMSO) to solubilize test chemicals, we tested for their effects on [3H]-digoxin accumulation compared to the no-addition control.

**Measurement of variability and a test for dietary alteration in Pgp activity in chickens**

We fed 12 chickens one of three different dietary treatments: corn-soy based diet containing no additions (control), 50 mg/kg rifampin (a known inducer of Pgp expression; Greiner et al. 1999; Geick et al. 2001) or 10 g/kg St. John's Wort (a known inducer of Pgp expression; Perloff et al. 2001), n = 4 birds/treatment.
After 7 days on the diet, sufficient time to induce Pgp expression based on cell-culture studies (Mickle et al. 1989; Bhat et al. 1995; Fardel et al. 1996; Perloff et al. 2001) and in vivo studies with rats (Salphati and Benet 1998; Sandstrom and Lennernas 1999), we euthanized birds and conducted everted-sleeve measurements as described above, making two replicate measures of digoxin accumulation with and without CsA (10 μM) in each bird. We calculated variation within birds, between birds, and within treatments for: (1) digoxin accumulation per milligram tissue (control and CsA 10 μM), and (2) our index of Pgp activity. We also tested for an effect of diet on the index of Pgp activity using the average within each bird of the two replicates of digoxin accumulation with and without CsA.

Effect of putative modulators of Pgp

To determine if common plant or fungal metabolites found in avian natural diets affect transport by Pgp, we tested genistein (GEN), an isoflavone found in soy and other legumes, quercetin (QUERC), a flavonoid ubiquitous in fruits, isouqueretin (ISOQ), the 3-β-glycoside of quercetin, and two fungal metabolites, aflatoxin-B1 (AFB1) and sterigmatocystin (ST), for their ability to affect accumulation of [³²H]-digoxin. Phytochemical concentrations were chosen based on previous studies in cell culture and tissue studies: quercetin (10–100 μM) and isouqueretin (100 μM) (Scambia et al. 1994; Critchfield et al. 1994; Shapiro and Ling 1997; Lo and Huang 1999; Mitsunaga et al. 2000), genistein (200 μM) (Versantvoort et al. 1993; Castro and Altenberg 1997), and aflatoxin-B1 (5 μM) (Burt and Thorgeirsson 1988; Santoni-Rugiu and Silverman 1997). There are currently no data on sterigmatocystin, so we matched the concentration to the closely related aflatoxin-B1 (5 μM). After eversion of the intestine, adjacent 1.5-cm sleeves were randomly assigned to one of five treatments (concentrations and vehicle controls reported in Results). Three same-aged chickens were tested on the same day.

Statistics

Numerical results are given as means ± SEM (n = sample size). To detect treatment effects, we preferred to include both treatment and control sleeves in the same animal and use Student’s paired t-tests or repeated-measures ANOVA, followed by planned contrasts. When we were unable to include all treatments and the control in the same animal (i.e., CsA dose response in chicken) or when we tested dietary induction, we used one-way ANOVA followed by post hoc Fisher’s LSD, when the global test resulted in P < 0.05. Treatment effects of plant and fungal metabolites in chicken were first analyzed with an incomplete-blocks ANOVA, then pair-wise comparisons were made with Student’s paired t-tests. We visually inspected plots of residuals to verify a random distribution. The P < 0.05 level was considered significant, while 0.05 < P < 0.10 was taken to indicate a trend. Analyses were done using Systat 10 and SAS.

Results

Effect of cyclosporin A on [³²H]-digoxin accumulation

Accumulation of tracer [³²H]-digoxin, corrected for adherent label, increased sublinearly with increasing concentration of CsA in the solution for intestinal sleeves from chicken (Fig. 1a), laboratory rat (Fig. 1b), and wild rodents (not shown). Cyclosporin A precipitated from the solution at around 15 μM, but [³²H]-digoxin accumulation at that saturating concentration

Fig. 1 Digoxin accumulation in mid-intestinal sleeves, pre-incubated for 2 min and incubated for 8 min, exposed to increasing concentration of cyclosporin A. a Broiler chicken (1, 3.3, 10, ~15 μM CsA), digoxin accumulation in the presence of CsA at all concentrations was significantly greater than control (one-way ANOVA, F₄,₅₅ = 19.1; P < 0.001; n = 9–12 sleeves/concentration). b Sprague-Dawley rat (1, 5, 10, ~15 μM CsA), digoxin accumulation in the presence of CsA (5, 10, 15 μM) was significantly greater than control (repeated-measures ANOVA, F₄,₅₅ = 19.2; P < 0.05; n = 3 sleeves/concentration). In both cases digoxin accumulation at 10 μM CsA was not significantly different from accumulation at 15 μM CsA. Points are mean ± SEM.
was not significantly higher than at 10 μM (chicken: one-way ANOVA, $F_{4.46} = 19.3; P < 0.001$, followed by pair-wise comparisons; rat: repeated-measures ANOVA, $F_{4.8} = 19.2; P < 0.001$, followed by pair-wise comparisons). Therefore, in subsequent experiments we used 10 μM CsA as a maximal inhibitory concentration, and calculated the index of Pgp activity as the difference between accumulation of [3H]-digoxin, in the presence and absence of 10 μM CsA, in adjacent intestinal tissues (i.e., digoxin excluded per mg wet tissue mass).

Although we assume that increased digoxin accumulation in the presence of CsA is due to competitive inhibition of digoxin’s exclusion by Pgp, it is also conceivable that CsA acts by depressing ATP generation, which powers the expulsion of compounds by Pgp (Ambudkar et al. 2003). To test for this nonspecific effect, we compared mediated uptake of 1 mM D-glucose, which is also powered by ATP, in the presence and absence of 10 μM CsA (0.1% ethanol as vehicle). We also included a measure of D-glucose uptake in the presence of phlorizin (1 mM), a potent inhibitor of the D-glucose transporter SGLT-1, to confirm that our measure was indeed of active D-glucose uptake. D-glucose uptake was not significantly depressed in the presence of CsA in either the lab rat (89 ± 16% of vehicle control) or chicken (90 ± 12%), but was significantly depressed in the presence of phlorizin (2 ± 1% in both species) (repeated-measures ANOVA with three chickens and four rats, $F_{2.10} = 13.7; P = 0.001$, followed by pair-wise comparisons).

Effect of incubation time on the measurement of Pgp activity

The incubation time should be long enough that uptake/accumulation is appreciable and accurately measurable, but not so long that tissue structural or functional integrity is compromised. Chicken intestinal sleeves were pre-incubated for 2 min and then incubated with and without 10 μM CsA for 4, 7, 8, 10, and 15 min. Tracer [3H]-digoxin accumulation in the presence of CsA, and in its absence (i.e., in ethanol vehicle control), increased with incubation time (Fig. 2a). The difference between the two, the index of Pgp activity, increased with increasing incubation time (all three regressions significant $P < 0.001$; $R^2 = 0.42, 0.73,$ and 0.58, respectively; $n = 3–6$ paired sleeves/time period) (Fig. 2a). Histology of chicken intestinal tissues incubated for 15 min showed signs of damage to villi. With rat intestine, we observed no tissue damage at incubation times up to 12 min (the longest time tested). In rats, there was no significant difference in tracer [3H]-digoxin accumulation with increasing incubation time in the presence or absence of 10 μM CsA, or the difference between the two over the small range of times tested ($P = 0.27, 0.10, 0.92$, respectively; $n = 2–7$ paired sleeves/time period) (Fig. 2b).

Reproducibility

To assess reproducibility, we compared the index of Pgp activity, which is the difference in tracer [3H]-digoxin accumulation in the presence and absence of 10 μM CsA, in adjacent pieces of mid-gut from the same chicken, and then compared the mean of the two values for different individuals. These comparisons were made for four chickens on each of three different diets. The coefficient of variation (C.V. = 100x SD/mean) for duplicated measures of accumulation during 8-min incubations averaged 15 ± 3% ($n = 24$ duplicated measures in 12 chickens), with no significant differences between measures made in the presence versus absence of CsA. The C.V. for duplicated measures of the Pgp index was twice as large ($P = 0.02$; average 32 ± 7%, $n = 12$ duplicated measures in 12 chickens, range 1–70%); and it did not differ significantly between diet groups. The
C.V. among animals within the three diet groups averaged 41 ± 11%. We discuss later how these patterns of variation inform experimental design. Mean Pgp activity differed significantly (one-way ANOVA, $F_{2,8} = 4.44$; $P = 0.05$) among the diets (Control diet, 0.23 ± 0.05 fmol digoxin excluded mg$^{-1}$; St. John’s Wort, 0.36 ± 0.07; rifampin diet, 0.44 ± 0.04) after one animal that was fed St. John’s Wort extract was removed after identification as a statistical outlier.

Putative modulators of Pgp activity

We tested five potential modulators of Pgp activity found in avian diets by measuring tracer $^{3}$H-digoxin accumulation in adjacent sleeves in the presence and absence of the modulator. The intestinal sleeves of the chickens were exposed to solutions containing vehicle control (0.1% ethanol or 0.5% dimethyl sulfoxide, DMSO) or one of five treatments in vehicle during 2-min preincubations and 8-min incubations (see Methods for concentrations). In preliminary experiments with chicken intestine, we found that ethanol and DMSO vehicles had no significant effect on $^{3}$H-digoxin accumulation (repeated-measures ANOVA, $F_{2,4} = 0.93$; $P = 0.47$). The overall test of natural metabolites on digoxin accumulation was significant (incomplete blocks ANOVA, $F_{3,23} = 10.1$; $P < 0.0001$). Genistein, from soybean, significantly increased digoxin accumulation (Student’s paired $t$ test, $t_4 = 4.91$; $P = 0.008$) (Fig. 3), as predicted for this isoflavone that is thought to interact with Pgp (Castro and Altenberg 1997). Sterigmatocystin was the only other compound that significantly altered digoxin accumulation (Student’s paired $t$ test, $t_9 = 4.07$; $P = 0.003$; Fig. 3). Because quercetin has been shown to both inhibit and enhance Pgp activity in the concentration range of 1–100 μM (Mitsunaga et al. 2000) we conducted a dose response with quercetin (not shown). We found no significant difference in digoxin accumulation among tissues exposed to 0, 10, 33, or 100 μM quercetin (repeated-measures ANOVA, $F_{3,6} = 1.23$; $P = 0.38$), and the highest dose of 330 μM damaged the tissues, as indicated by PEG marker space measurements four times normal and corresponding negative values for accumulation.

Increased digoxin accumulation in the presence of genistein and sterigmatocystin might be due to competitive inhibition of digoxin’s exclusion by Pgp, but it is also conceivable that they act by depressing ATP generation, which powers the expulsion of compounds by Pgp (Ambudkar et al. 1999). To test for this nonspecific effect, we compared mediated uptake of 1 mM d-glucose, which is also powered by ATP generation, in the presence and absence of the two treatments. We also tested for effects of the vehicle control (DMSO) in a similar fashion. We maintained total exposure of tissue to heated Ringer solution by pre-incubating for 6 min and incubating with $^{3}$H-d-glucose for 4 min with or without test chemicals. $^{3}$H-d-glucose uptake was not significantly depressed in the presence of DMSO (86 ± 12% of ethanol control), sterigmatocystin (86 ± 17%) or genistein (83 ± 14) (repeated-measures ANOVA, $F_{3,15} = 1.18$; $P = 0.35$).

Interspecific comparisons of Pgp activity

We have applied this methodology to intestinal sleeves from four mammalian and two avian species (Table 1). In all species, 10 μM CsA significantly enhanced tracer $^{3}$H-digoxin uptake while having no significant effect on mediated d-glucose uptake (as described for Fig. 1), implying functional presence of Pgp. Indeed, in all the species tested, we have detected expression of a Pgp homolog in intestinal mucosa (data not shown; Barnes 2001). Villus structure was maintained during tissue incubations in all cases but one, the American robin (Turdus migratorius). The Sprague-Dawley rat exhibited the lowest Pgp activity of the species tested. Future studies might also compare among species the total Pgp capacity, by measuring Pgp activity along the entire small intestine length, because it may vary with intestinal position (Makhey et al. 1998; Brady et al. 2002). For example, in Sprague-Dawley rats and Stephens woodrats, a specialist on leaves of juniper, we measured Pgp activity along the gut by measuring tracer $^{3}$H-digoxin accumulation during 12-min incubations in adjacent intestinal sleeves from proximal-, mid-, and distal regions, in the presence or absence of 10 μM CsA (Fig. 4). Due to difficulty evertting the thick proximal region, we did not measure variation along the chicken intestine. There was a trend for lower Pgp activity in distal sections from Neotoma stephensi (repeated-measures ANOVA, $F_{2,8} = 3.28$, $P = 0.09$), and in Sprague-Dawley rats variation along the intestine was significant (repeated-measures ANOVA, $F_{2,8} = 4.46$; $P = 0.05$). Pgp

![Fig. 3 Effect of natural plant (GEN genistein, ISOQ isouqueretin, QUERC quercetin) and fungal (AFBI aflatoxin-B1, ST sterigmatocystin) metabolites on digoxin accumulation in broiler chicken jejunum tissue ($n = 3$–9 chickens). Values are mean ± SEM, † indicates treatment significantly different from control with $P < 0.01$ in a Student’s paired $t$-test following a significant incomplete blocks ANOVA ($F_{3,23} = 10.1$; $P < 0.0001$).](image-url)
Table 1 Measures of digoxin accumulation in the presence of vehicle (control), 10 μM CSA (CsA), and the difference in accumulation between CsA and control (Pgp activity index)

<table>
<thead>
<tr>
<th>Species</th>
<th>Control (fmol digoxin/mg tissue)</th>
<th>CsA (fmol digoxin/mg tissue)</th>
<th>Pgp activity index (fmol digoxin excluded/mg tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td>8 min</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Broiler chicken</td>
<td>0.230 ± 0.045</td>
<td>0.614 ± 0.151*</td>
<td>0.307 ± 0.030 (b)</td>
</tr>
<tr>
<td>RexW chicken</td>
<td>0.416 ± 0.175</td>
<td>0.686 ± 0.178*</td>
<td>0.270 ± 0.058 (b)</td>
</tr>
<tr>
<td>Sprague-Dawley rat</td>
<td>0.086 ± 0.051</td>
<td>0.159 ± 0.084*</td>
<td>0.073 ± 0.013 (a)</td>
</tr>
<tr>
<td>American robin</td>
<td>0.370 ± 0.035</td>
<td>0.429 ± 0.062</td>
<td>NSa</td>
</tr>
<tr>
<td>12 min</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sprague-Dawley rat</td>
<td>0.114 ± 0.078</td>
<td>0.169 ± 0.089*</td>
<td>0.059 ± 0.010 (a)</td>
</tr>
<tr>
<td>Neotoma stephensi</td>
<td>0.161 ± 0.018</td>
<td>0.302 ± 0.022*</td>
<td>0.334 ± 0.095 (b)</td>
</tr>
<tr>
<td>Neotoma albigena</td>
<td>0.211 ± 0.016</td>
<td>0.545 ± 0.085**</td>
<td>0.141 ± 0.026 (a)</td>
</tr>
<tr>
<td>Neotoma cinerea</td>
<td>0.161 ± 0.019</td>
<td>0.296 ± 0.036**</td>
<td>0.135 ± 0.038 (a)</td>
</tr>
</tbody>
</table>

For each incubation time (8 or 12 min), there were significant differences between species in the Pgp activity index (P < 0.05 by ANOVA), and the values that differ from each other (P < 0.005 with Fisher's LSD) are designated by different letters in parentheses (a or b) adjacent to the values.

Treatment effect of CsA within species using one-tailed t-test indicated by *significance at P = 0.001, **significance at P = 0.01. * Tissues incubated in 15 μM CsA. Histology showed structural damage to villi which may explain the lack of a significant (P = 0.055) Pgp activity index.

156 ± 11 cm/kg⁻³/µm [n = 5]; one-way ANOVA, F₁,₈ = 5.64, P = 0.045 and a heavier small intestine than woodrats (respectively, 24.5 ± 0.2 g/kg⁻³/µm [n = 5] vs. 12.4 ± 1.3 [n = 5]; one-way ANOVA, F₁,₈ = 87.7; P < 0.001).

Discussion

Validation of many aspects of measuring uptake by everted intestinal sleeves has been presented elsewhere (Karasov and Diamond 1983; Starck et al. 2000). We used longer incubation times (8–12 min) than are generally used for measuring sugar and amino acid uptake (generally 1–4 min) because our probe, [³H]-digoxin, is passively absorbed relatively slowly, and the target of our measurements is the difference in digoxin accumulation in the presence and absence of a competitor, reflecting digoxin excluded by Pgp. The difference in accumulation in the presence and absence of a competitor was not significant over very short incubation times but was significant over incubations of at least 8 min. As a competitor we used cyclosporin A, a well-known substrate and competitive inhibitor of Pgp (Lan et al. 1996), and, as expected, incubation with increasing concentrations of CsA caused a significant increase in [³H]-digoxin accumulation that reached an asymptote (at 10 μM) consistent with saturable competitive inhibition. Barthe et al. (1998) used everted rat intestinal sacs (2.5 cm long) to measure digoxin accumulation in the presence and absence of other competitive inhibitors (100 μM verapamil, 1.3 mM quinidine), but used much longer incubation times (15–90 min) to measure a difference. An advantage of our technique is that we can measure accumulation in smaller sections of intestine (1 vs. 2.5 cm) in a shorter period (8–12 vs. 15–90 min). This difference allows us to make more measures within an individual, keep treatment and control sections in close proximity, and keep total experiment time to a minimum (approx. 3 h from euthanasia through last
incubation). Furthermore, using smaller intestinal sections is an advantage in vertebrates where intestinal length is much smaller than the Sprague-Dawley rat or broiler chicken.

Because we are unable to directly measure production of a product as is often done in studies of enzyme activity, we must define a measure that can be used to compare among individuals, populations, and species. We used the difference in digoxin accumulation between tissues exposed to CsA (10 μM) and vehicle control as an index of Pgp activity. This is an index because we must assume that this value represents the amount of a compound that would enter a tissue if Pgp were not present. We also must assume that CsA and digoxin interact with Pgp in the same way among different individuals, populations, and species. At this time, we are unable to test these assumptions and we must, therefore, interpret our data with caution.

Due to the increased incubation time over typical nutrient uptake experiments (Karasov and Diamond 1983), we repeated some tests of condition and viability. Observation of the histology of everted sleeves showed that villi remained intact in laboratory rats, chickens, and woodrats throughout the experimental procedure with 2-min pre-incubations and incubations up to 12 min. Damage was evident in tissues from American robins, which is why we made only limited measures on this species. D-glucose was actively transported by tissues during the incubation time periods used here indicating functional integrity, and none of the test compounds that altered [3H]-digoxin accumulation (CsA, genistein, sterigmatocystin), or served as vehicles for those test compounds (DMSO), significantly depressed the mediated glucose transport. Thus, incubations of 8–12 min are long enough that [3H]-digoxin accumulation is appreciable, making Pgp activity accurately measurable, but not so long that structural or functional integrity of the tissue is compromised in most species. Incubations of 8–12 min are also short enough for one investigator to test a dozen tissues from an animal within 3 h post-dissection, which also helps insure tissue viability (Karasov and Debnam 1987).

Replicated measures of [3H]-digoxin accumulation by adjacent tissues within individuals had larger coefficients of variation (C.V.; average 15% of means) than were reported for d-glucose uptake (range 5–9%; Karasov and Diamond 1983), perhaps because of slower uptake of digoxin. Not surprisingly, the C.V. for replicated measures of Pgp activity was even larger (32%) because that measure is based on the difference between two measures of accumulation that each contribute variation. Simple power calculations indicate that sample sizes of six per treatment group would be necessary to discriminate a doubling in Pgp activity (for α = 0.05, β = 0.8). Smaller sample sizes might be used to test putative competitors by comparing [3H]-digoxin accumulation in adjacent tissues, which have a smaller C.V.

The methods we describe can be used to study alterations in Pgp activity within and between species over the short time scales of individual meals to the longest time scales of evolutionary time. Among the few species that we have studied (Table 1), Pgp activity per unit tissue varies by three to four times, and by two times at the whole animal level once corrections are made for differences in body size. What are the ecological consequences and evolutionary causes of such differences? In marine and freshwater mollusks, considerable variation in Pgp-like activity has been demonstrated among populations within species and between species (Epel 1998), and there are positive relationships between activity and species-specific tolerance to organic pollution (Smital et al. 2000; Bard 2000). The mollusk species with the highest level of Pgp-like activity were taken from sites that were polluted with industrial and household wastes for many years, those with intermediate levels were taken from less polluted sites, and the lowest basal activity was found in species collected only from unpolluted sites. Smital et al. (2000) suggested that the inherent level of Pgp-like activity could be a result of selection pressure during the life history of the species, subspecies, or even different populations of the same species. These findings in invertebrates beg the question of whether differences among vertebrate species in constitutive levels of Pgp activity could have arisen due to natural selection in environments (e.g., diets) that differ in levels of natural and manmade toxins. The methods described here can be used to test for such patterns in a wide variety of vertebrate species.

Besides differences in constitutive levels, differences between populations can arise due to modulation of activity within individuals. In our study, chickens that were fed a basal diet containing rifampin for 7 days had jejunal Pgp activity approximately twice that of those that were fed the basal diet, consistent with findings in laboratory rats provided rifampin orally (Sandstrom and Lennernas 1999). Much remains to be learned about Pgp’s role in the flexible biochemical responses to ingested toxins of species that regularly or irregularly consume diets high in potential toxins, either manmade or natural.

The extent to which constitutive or induced levels of intestinal Pgp will retard absorption of natural or anthropogenic toxins can depend on the presence of other dietary constituents (Phang et al. 1993; Soldner et al. 1999). In our study with chicken intestine, the presumed Pgp-mediated inhibition of [3H]-digoxin absorption was significantly reduced by the presence of a plant metabolite, genistein, or fungal metabolite, sterigmatocystin, in the incubation solution (Fig. 4). The simplest mechanistic explanation is competition for Pgp binding site(s) between [3H]-digoxin and genistein, as has been seen previously (Castro and Altenberg 1997; Conseil et al. 1998), or sterigmatocystin. Another plausible mechanism is depletion of cellular ATP upon which Pgp activity relies, but this explanation seems less plausible based on the absence of a nonspecific effect of either genistein or sterigmatocystin on active d-glucose absorption. Quercetin, a ubiquitous flavonol found in
many fruits and vegetables either glycosylated or as an aglycone, was reported to deplete ATP levels in tumor cells (Versantvoort et al. 1993), decrease ATPase activity (Shapiro and Ling 1997), and to interact with substrate and ATP binding site(s) in purified Pgp (Shapiro and Ling 1997; Conseil et al. 1998). However, in our tests with isolated chicken intestine, neither quercetin over a wide range of concentrations, nor isoquercetin (the 3-β-glycoside of quercetin), had a significant effect on [3H]-digoxin accumulation.

Although cell lines and vesicle preparations that over-express Pgp are useful for demonstrating the potential of dietary compounds to interact with Pgp, the response of intact tissue in vitro or in situ may offer additional insight into the likely significance of the interaction in a nutritional context. This advantage, along with the aforementioned opportunities to study alterations in Pgp activity within and between species, supports the inclusion of the methods that we have described among the repertoire of techniques for studying the physiological and ecological significance of Pgp.

Acknowledgements We would like to thank B. Darken for extensive help in the lab. Support for this research came from USDA (Hatch) WISO4322, NSF IBN-9723793 and IBN-0216709 to W.H.K. A.K.G was supported by an NSF pre-doctoral fellowship and a UW-Madison graduate fellowship. All research conformed to UW-Madison IACUC protocols.

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