Induction of Rat Intestinal P-glycoprotein by Spironolactone and Its Effect on Absorption of Orally Administered Digoxin

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ABSTRACT

The effect of the diuretic spironolactone (SL) on expression and function of intestinal P-glycoprotein (P-gp), as well as its impact on intestinal absorption of digoxin, was explored. Rats were treated with daily doses of 200 \( \mu \text{mol/kg b.wt.} \) of SL intraperitoneally for 3 consecutive days. The small intestine was divided into four equal segments of \( \sim 25 \text{ cm} \), with segment I being the most proximal. Brush-border membranes were isolated and used in analysis of P-gp expression by Western blot analysis. P-gp content increased in the SL group by 526, 292, 210, and 622% over controls for segments I, II, III, and IV, respectively. Up-regulation of apical P-gp was confirmed by immunofluorescence microscopy. P-gp transport activity was explored in intestinal sacs prepared from segment IV using two different model substrates. Serosal to mucosal transport (efflux) of rhodamine 123 was 140% higher, and mucosal to serosal transport (absorption) of digoxin was 40% lower in the SL group, both indicating increased P-gp function. In vivo experiments showed that intestinal absorption of a single dose of digoxin administered p.o. was attenuated by SL pretreatment. Thus, concentration of digoxin in portal and peripheral blood was lower in SL versus control groups, as well as its accumulation in kidney and liver. Urinary excretion of digoxin was significantly decreased in the SL group, probably reflecting decreased systemic availability of digoxin for subsequent urinary elimination. We conclude that SL induces P-gp expression with potential impact on intestinal absorption of substrates with therapeutic application.

P-glycoprotein (P-gp) is a versatile xenobiotic pump, which was first described in cancer cells, decreasing accumulation of chemotherapeutic agents such as the vinca alkaloids. It is constitutively expressed in a variety of normal human and rodent tissues, including liver, brain, adrenal gland, kidney, and intestinal tract epithelia. In the small intestine and colon, P-gp is one of the most important efflux proteins (Sun et al., 2004). Localized to the apical membrane of the mature epithelial cells, it is responsible for pumping a wide range of xenobiotics, mostly hydrophobic, into the intestinal lumen. P-gp is encoded by the multidrug resistance gene \textit{MDR1} in humans and by genes \textit{Mdr1a/b} in rats, and because of its high expression in intestine, P-gp probably plays a physiological role as a defense mechanism against toxic substances present in the diet.

Several studies report on the role of intestinal P-gp in modulating oral drug bioavailability (Fromm, 2003; Lin and Yamazaki, 2003). In addition, P-gp is known to be induced in vitro and in vivo by a wide range of xenobiotics and therapeutic drugs, hormones, and classical inducers, either in experimental animals or humans (Schuetz et al., 1996; Takano et al., 2006). Among these compounds, the effect of rifampicin has been well documented by different groups. During rifampicin treatment, plasma concentrations of several p.o. administered therapeutic drugs, all shown to be P-gp substrates, are decreased (Takano et al., 2006). As a consequence, a decreased pharmacological potency of these drugs is expected. Clearly, potential drug interactions should be considered when P-gp inducers/substrates are coadministered clinically.

Spironolactone (SL), an aldosterone antagonist, is a widely used diuretic indicated in patients with edema or ascites produced by cirrhosis or congestive heart failure (Ochs et al.,

ABBREVIATIONS: P-gp, P-glycoprotein; SL, spironolactone; BBM, brush-border membrane; i.p., intraperitoneal.
Materials and Methods

Chemicals. [3H]Digoxin (37.0 Ci/mmol) and OptiPhase liquid scintillation mixture were purchased from PerkinElmer Life Science Products (Boston, MA). Unlabeled digoxin was from ICM Biomedicals Inc. (Costa Mesa, CA), and SL, verapamil, leupeptin, phenylmethylsulfonyl fluoride, pepstatin A, and bovine serum albumin were purchased from Sigma Chemical Co. (St. Louis, MO). All of the other chemicals were of analytical grade purity.

Animals and Treatment. Male Wistar rats (250–290 g) were used throughout. They were maintained ad libitum on a standard laboratory pellet diet and were allowed free access to water and saline during treatment. A group of animals was treated with SL (200 μmol/kg i.p.) in propylene glycol (60 mM) or vehicle alone (3.3 μmol/kg) for 3 consecutive days. This dose was shown to maximally induce microsomal UDP-glucuronosyltransferase (Mottino et al., 1989) and canalicular multidrug resistance-associated protein 2 in rat liver (M. L. Ruiz, S. S. M. Villanueva, A. D. Mottino, and V. A. Catania, unpublished results). Studies were performed 18 h after the last injection of SL or vehicle. All of the procedures were conducted in accordance with National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

Specimen Collection and Plasma Membrane Preparation. Rats were anesthetized with sodium pentobarbital (50 mg/kg i.p.), and the whole small intestine was removed and divided into four equal segments, which were carefully rinsed with ice-cold saline. The proximal segment, starting from the pylorus, was given the number I, whereas the most distal segment, close to the ileocecal valve, was given the number IV. These segments were immediately used in crude plasma membrane preparation as described previously (Mottino et al., 2000). Livers were perfused with ice-cold saline through the portal vein, removed, and immediately used in crude plasma membrane preparation as described previously (Meier et al., 1984). Protein concentration in membrane preparations was measured using bovine serum albumin as a standard (Lowry et al., 1951).

Western Blot Studies. Immunoblotting for detection of P-gp was performed using a rabbit polyclonal antibody to human MDR-1 (Santa Cruz Biotechnologies, Santa Cruz, CA) as described previously (Ghanem et al., 2004). Densitometry was performed using the Gel Pro Analyzer (Media Cybernetics, Inc., Silver Spring, MD) software.

Immunofluorescence Microscopy Studies. Slices (5 μm) from distal ileum were prepared with a Zeiss Microm HM500 microtome cryostat (Zeiss, Walldorf, Germany), air-dried for 2 h, and fixed for 10 min with ice-cold acetone (−20°C). For labeling, tissue sections were incubated overnight with the polyclonal anti-human MDR-1 (1:100) antibody. Sections were then washed five times with phosphate-buffered saline and incubated with Cy3-conjugated donkey anti-rabbit immunoglobulin G (Jackson ImmunoResearch Laboratory, Inc., West Grove, PA) (1:200) for 2 h. After washing three times with phosphate-buffered saline and once with distilled water, slices were air-dried and mounted. The images were captured on a Zeiss Axiovert 25 CFL inverted microscope (Zeiss, Gottingen, Germany). To ensure comparable staining and image capture performance for SL and control groups, intestine slices were prepared the same day, mounted on the same glass slide, and subjected to the staining procedure and microscopy analysis simultaneously.

P-gp Activity in Intestinal Sacs. Intestinal sacs were prepared from segment IV, which was removed as described above. P-gp activity was examined analyzing the serosal to mucosal transport (efflux) of rhodamine 123, a well-known P-gp substrate. Four-centimeter segments were filled (mucosal compartment) with Krebs-Henseleit buffer (40 mM glucose, pH 7.4) previously gassed with 95% O2/5% CO2. After 15 min of preincubation in 100 μl of the same buffer at 37°C (serosal compartment), with or without 100 μM verapamil, a P-gp inhibitor (Zhang et al., 2005), rhodamine 123 was added to this external medium to reach a final concentration of 50 μM. The sacs were incubated for 0, 5, 10, 20, or 40 min. The external medium was continuously gassed with 95% O2/5% CO2. At the end of incubation, the exterior of the sacs was carefully rinsed with drug-free buffer, and the entire mucosal solution was collected. Intestinal tissue was homogenized in 2.5 ml of the same buffer. Rhodamine 123 concentration was determined spectrophotometrically (Effert et al., 1989) in aliquots of serosal and mucosal buffers and in intestinal homogenates. The fluorescence intensity of rhodamine 123 was measured at an excitation wavelength of 488 nm and emission wavelength of 550 nm.

Intestinal Absorption of Digoxin. Intereference of intestinal P-gp induction on intestinal absorption was evaluated in vitro and in vivo.

Absorption in Intestinal Sacs. Four-centimeter segments isolated from distal ileum (segment IV) were everted and filled with Krebs-Henseleit buffer (serosal compartment). Everted sacs were preincubated in 100 μl of buffer (mucosal compartment) for 15 min in the presence or absence of 100 μM verapamil. Digoxin was then added to the external medium to reach a final concentration of 10 μM (10.6 μmol of [3H]digoxin/mol of unlabeled digoxin). Incubation was performed as described above. Radioactivity was determined in serosal and mucosal samples and in intestinal homogenates by liquid scintillation analysis (Wallac counter 1409; Turku, Finland).

Absorption and Distribution of Digoxin in Vivo. Digoxin absorption and subsequent distribution into blood, liver, and kidney were examined by analyzing its portal concentration, as well as its systemic blood concentration and hepatic and renal content and disposition. Rats were anesthetized with sodium pentobarbital (50 mg/kg i.p.) and thus maintained throughout the experiment. Body temperature was measured with a rectal probe and maintained at 37°C with a heating lamp. Appropriate volumes of 5% bovine serum albumin in saline were administered i.v. throughout the experiment to replenish body fluids. To study digoxin systemic distribution, the jugular vein and the carotid artery and common bile duct were cannulated with polyethylene tubing (PE50 and PE10, respectively). The urinary bladder was exteriorized through an abdominal midline incision and cannulated with polyethylene tubing (PE75). The animals received a p.o. dose of digoxin of 25.6 μmol/kg (17.0 μmol of [3H]digoxin/mol of unlabeled digoxin) by gavage, and arterial blood,
bile, and urine were sampled at 15-min periods for 135 min. At the end of the experiment, the liver and kidneys were removed and homogenized in 20 and 6 ml of saline, respectively. Bile and urinary flow were determined gravimetrically. Samples of serum, bile, urine, and homogenates were used for assessment of digoxin concentration by liquid scintillation analysis. To study the portal content of digoxin, PE10 tubing was inserted into the portal vein and secured with an adhesive agent in a different set of animals. Portal blood flow was not altered by this procedure. The jugular vein was cannulated to replenish body fluids as described above. A dose of 25.6 nmol/kg h.wt. of digoxin (17.0 µmol of [3H]digoxin/mol of unlabeled digoxin) was administered p.o. by gavage, and blood from the portal vein was sampled at 15, 20, 25, and 30 min after digoxin administration. Serum samples were used for determination of digoxin concentration by liquid scintillation analysis.

**Statistical Analysis.** Data are presented as the mean ± S.D. Comparison between groups was performed using the Student’s t test or one-way analysis of variance followed by Bonferroni test (when more than two groups were compared). Values of P < 0.05 were considered to be statistically significant.

**Results**

**Effect of SL on P-gp Expression.** Because P-gp expression varies along the small intestine (Doherty and Charman, 2002), we divided the whole organ in four segments of the same length. Figure 1A shows Western blot detection of P-gp in BBM isolated from SL and control groups in these different regions. SL induced an increase in P-gp expression of 526, 292, 210, and 622% over controls for segments I, II, III, and IV, respectively. To confirm the localization of P-gp in situ, we performed an immunofluorescence microscopy study. Figure 1B shows that P-gp is mainly localized to the surface of the intestinal villus and that increased fluorescent detection of this transporter is seen in the SL group compared with

![Image](image_url)
controls. In some regions of the villus, detection of P-gp is clearly evidenced at the apical membrane, particularly in the SL group (see arrowheads).

P-gp is widely expressed in different tissues. To evaluate whether SL induction of P-gp was restricted to the small intestine, we analyzed its expression in crude plasma membranes from liver. Figure 1C shows that P-gp expression was increased in liver of SL animals, although to a less extent than in intestine. By densitometry, detection of P-gp protein was increased by 40% in the SL group.

**Effect of SL on P-gp Transport Activity.** To determine the functional impact of increased P-gp protein levels by SL, we studied the secretion of rhodamine 123, a typical P-gp substrate, into the mucosal compartment of intestinal sacs prepared from distal ileum. Addition of rhodamine 123 to the serosal compartment resulted in progressive recovery of the dye in the mucosal side. Figure 2A shows that basolateral to apical transport of rhodamine 123 was increased by SL. Efflux of the dye was 140% higher in the SL group at 40 min. It is known that rhodamine 123 is also a substrate for breast cancer resistance protein (Bcrp or Abcg2) (Alqawi et al., 2004), which is expressed in intestine from rodents (Han and Sugiyama, 2006). To further confirm participation of P-gp in transport of rhodamine 123 (see Fig. 2B), thus suggesting that the difference in the secretory rate of the dye produced by SL pretreatment was caused by a higher transport efficiency at the apical membrane, and not by a different uptake at the basolateral membrane.

**Effect of P-gp Induction on Intestinal Absorption of Digoxin.** P-gp in intestine functions as a membrane barrier against absorption of xenobiotics and drugs available intraluminally. To test whether SL also enhances this particular function, we first evaluated the mucosal to serosal transport of digoxin, a typical substrate of P-gp (Drescher et al., 2003), in everted intestinal sacs. Figure 3A shows that intestinal absorption of digoxin was 40% lower in SL versus controls after 40 min of incubation. In the presence of verapamil, both groups showed an increase in the absorption of digoxin (2.6- and 2.0-fold for SL and control groups, respectively) with respect to incubations without inhibitor. These results clearly implicated P-gp as a membrane barrier for restriction of digoxin absorption. The intestinal content of digoxin in the SL group was significantly lower than in the control group (see Fig. 3B). However, when P-gp activity was assayed in the presence of verapamil, the intestinal concentration of...
digoxin was similar in both groups. Taken together, these
data indicate that induction of apical P-gp has led to
decreased intracellular availability of digoxin for subsequent
secretion into the serosal compartment, thus explaining the
lower rate of mucosal to serosal transport shown in Fig. 3A.

Intestinal P-gp was described to be one of the factors that
modulate digoxin steady-state concentrations (Nakamura et
al., 2001). Variations of serum concentration of digoxin with
time after its p.o. administration are depicted in Fig. 4A.
Whereas digoxin concentration increased with time in the
control group, this measure remained constant in the SL
group; a statistically significant difference was observed be-
tween groups from 90 min onward. SL was found to induce
hepatic P-gp in addition to intestinal P-gp. To confirm that
the difference observed in digoxin serum concentration be-
tween groups was caused by a decrease in the intestinal
absorption of the drug and not by an increase in hepatic
first-pass extraction and elimination, we evaluated the con-
centration of the drug in portal vein. The results shown in
Fig. 4D show a significant reduction in digoxin concentration
25 and 30 min after drug administration.

As a consequence of its decreased intestinal absorption,
digoxin exhibited reduced accumulation in liver and kidney
from SL rats (−65 and −88%, respectively; see Fig. 4C) 135
min after drug administration. Figure 4B shows that cumu-
lative biliary excretion of digoxin was preserved in SL ani-
imals and that cumulative urinary excretion was decreased
by 88%. The first-pass effect in which the liver extracts most
of the drug absorbed probably contributed to the decreased
urinary excretion of digoxin, whereas biliary excretion was
maintained.

Discussion

Because of the central role that intestinal P-gp plays in the
absorption and presystemic elimination of many chemicals,
including medicines, understanding the factors that regulate
its expression is relevant both from a clinical and toxicolog-
ic point of view. In this study, we evaluated the effect of
pretreatment with SL on the expression and activity of in-
testinal P-gp in the rat. We chose this known inducer of
multiple enzyme systems because it presents a therapeutic
application as a diuretic and is often coadministered with
digoxin, a typical P-gp substrate, in patients with congestive
heart failure. Thus, drug-drug interactions may occur. The
current data indicate significant induction of intestinal P-gp
by SL. Although the mechanism by which SL modulates
expression of this protein is unknown, because SL is a well
known ligand for pregnane X receptor (Schuetz et al., 1998),
it is possible that binding of SL to this nuclear receptor
represents the initial step in P-gp induction.

We showed that the increase in expression of P-gp at the
apical membrane of the enterocyte correlated well with in-
creased transport activity detected in in vitro experiments
using two different P-gp substrates: rhodamine 123 and
digoxin, the latter of therapeutic application. Digoxin is
widely used in the treatment of congestive heart failure, and
it is important to note that this drug presents a narrow
therapeutic index. Increased plasma levels could result in
dangerous toxicity to the heart, whereas a decrease in its
blood concentration would produce an ineffective treatment.
For example, coadministration of quinidine (Fromm et al.,
1999; Verschraagen et al., 1999), verapamil (Verschraagen et
al., 1999), or diltiazem (Takara et al., 2002) with digoxin
results in increased digoxin plasma levels, most likely as a
consequence of inhibitory action on intestinal P-gp. Likewise,
it is expected that modulation of P-gp expression affects digoxin absorption and disposition. To test the possibility that SL induction of intestinal P-gp affects digoxin absorption, we further performed experiments in vivo. Analysis of digoxin portal concentration versus time showed a decrease in intestinal absorption of this drug after its p.o. administration in the SL group. This likely resulted in decreased systemic concentration of digoxin, as revealed by detection of lower plasma, renal, and liver contents. Renal elimination of digoxin depends on a balance between filtered and tubular processes. Although the mechanism for impaired urinary excretion of digoxin in the SL group was not explored, the data on decreased systemic and portal plasma concentrations strongly suggest decreased systemic availability for subsequent renal elimination. Interestingly, we found no change in biliary elimination of digoxin despite decreased plasma and intrahepatic concentration of the drug. This result can be tentatively explained by a compensatory increase in expression of liver P-gp as detected by Western blot analysis, additionally contributing to decreased digoxin intracellular levels. Taken together, these data suggest lower tissue digoxin exposure in SL rats, mainly as a result of increased intestinal barrier function against absorption of selective P-gp substrates.

Previous studies in experimental animals showed that treatment with SL decreases toxicity of digitoxin (Buck and Lage, 1971; Solymoss et al., 1971), another P-gp substrate with similar therapeutic application as digoxin. Digitoxin is partially metabolized to digoxin by hepatic microsomal enzymes (Eberhart et al., 1992), undergoes efficient enterohepatic recirculation, and only a small fraction of unchanged drug is eliminated in feces either in humans or rats (Rietbrock and Vohringer, 1974; Vohringer and Rietbrock, 1974). Protection against digitoxin toxicity by SL has been ascribed to hepatic SL-induced metabolism of the drug (Selye et al., 1969; Buck and Lage, 1971; Solymoss et al., 1971). According to the current results, it is also possible that modulation of digitoxin toxicity by SL results from increased elimination of the intact drug or its metabolite digoxin. Our Western blot study showed a substantial increase in expression of P-gp not only in distal but also in proximal intestine, where P-gp constitutive expression is normally low. In consequence, the extended induction of the transporter, shown in this work, may critically affect absorption of digitoxin or its metabolite digoxin, either from oral or biliary source, once enterohepatic recirculation is completed. This could result in shortening the drug half-life and decreasing the toxicity, as previously observed.

Wirth et al. (1976) reported the effect of SL on the metabolism of i.v. administered [3H]digoxin in patients receiving SL chronically. SL caused a 20% reduction in the half-life of serum radioactivity and a 16% reduction in the volume of distribution. Induction of phase II (e.g., UDP-glucuronosyltransferase) enzymes by SL was proposed to explain these alterations, which seem to contribute to the reduction in half-life. The dose of SL administered to these patients (100–200 mg/day) is proportionally far below the dose administered to rats in the current study. However, the therapeutic protocol requires chronic administration and could represent a much more sustained action leading to alterations not only in metabolism but also in transport of drugs coadministered with SL.

In conclusion, the present study shows that SL increases the expression and activity of P-gp throughout the small intestine in the rat. These modifications have pronounced effects on in vivo intestinal absorption of digoxin when administered p.o. The possibility that simultaneous treatment with SL and digoxin results in clinical drug-drug interactions is suggested.

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