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DRUG DEVELOPMENT AND INDUSTRIAL PHARMACY®
Vol. 29, No. 7, pp. 767–775, 2003

RESEARCH PAPER

Effect of Sodium Alterations on Hepatic Cytochrome P450 3A2 and 2C11 and Renal Function in Rats

Jinrong Liu, Shellie M. Callahan, and Lane J. Brunner*

Pharmaceutics Division, College of Pharmacy, The University of Texas at Austin, Austin, Texas, USA

ABSTRACT

Numerous dietary supplements are known to modulate cytochrome P450 (CYP)-mediated metabolism and subsequently alter drug toxicity or efficacy in animals and humans. In the present study we investigated the effect of varying amounts of sodium intake on renal function and the metabolic activity of the hepatic CYP3A2 and CYP2C11 isoforms. Rats were maintained on standard rodent chow or a low-salt rice diet. Within each of these groups rats received either a single intraperitoneal injection of furosemide to initiate salt depletion, or saline. Additional groups included salt supplementation of 500 mg/300 g body weight/day and 1.25 g/300 g body weight/day of sodium chloride solution. Rats receiving the low-salt diet, both with and without a concomitant furosemide administration, had a significant reduction in creatinine clearance without changes in serum creatinine. In addition, urine flow rate was markedly reduced in rats maintained on the low-salt diet. Western blot analysis indicated that neither sodium supplementation nor deprivation altered hepatic microsomal CYP3A2 levels; however, hepatic CYP2C11 levels significantly increased in rats receiving the largest sodium supplement. In vitro metabolic activity of CYP3A2 was unchanged as compared with controls. Activity of CYP2C11 was significantly reduced in both rat groups receiving additional sodium supplements. Acute manipulation of daily sodium intake does alter renal function and specific hepatic CYP isoforms and should be considered when using these rat models.

Key Words: CYP; Hepatic; Rat; Renal function; Sodium.

*Correspondence: Lane J. Brunner, Pharmaceutics Division, College of Pharmacy, The University of Texas at Austin, Austin, TX 78712-1074, USA; Fax: (512) 471-7474; E-mail: lane.brunner@mail.utexas.edu.

DOI: 10.1081/DDC-120021776
0363-9045 (Print); 1520-5762 (Online)

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INTRODUCTION

Cytochrome P450s (CYP) are a superfamily of genes whose enzymes represent a diverse group of isoforms related by amino acid sequence identity and substrate specificity.[1] Currently there are more than 480 different isoforms encoded by more than 70 different gene families in 85 eukaryotic and 20 prokaryotic species. Although CYP enzymes have been found in numerous tissues, those found in the liver, kidney, and small intestine have received the greatest interest regarding drug metabolism and related effects on drug therapy. The enzymes from the CYP3A subfamily are some of the most abundantly found, in both human and rat hepatic microsomes. While CYP3A4 is the predominant CYP3A isoform in humans, CYP3A2 performs similar functions in rats. The sequences of these two genes are 72% homologous.[2] A second major hepatic CYP isoform is CYP2C11. It has been estimated that CYP2C11 accounts for up to 50% of all hepatic CYP enzymes in the rat.[3]

Diet and nutrition are widely recognized as modulating factors in the metabolism of xenobiotics.[4–6] This modulation by dietary factors is primarily through the induction or inhibition of specific hepatic CYP isoforms. Thus, dietary factors may have marked effects on the metabolism of therapeutic agents, possibly resulting in alterations in drug efficacy. Bailey et al. reported that grapefruit juice decreases the metabolism of the calcium channel antagonist, felodipine, and its primary metabolite, dehydrofelodipine, thus prolonging the effective concentration of the drug.[7] In addition, concurrent ingestion of grapefruit juice has been shown to decrease the absorption and metabolism of the immunosuppressant, cyclosporine, in both normal volunteers[8] and transplant patients.[9] The increased drug levels are the result of metabolic inhibition of gastrointestinal and hepatic metabolism by flavonoids[10,11] or alternatively, induction of the multidrug efflux pump, P glycoprotein, in the small intestine enterocytes.[12,13]

In addition to diet and nutrition, dietary vitamin and mineral homeostasis is significant in the regulation of drug metabolism. Martini and Murray reported that rats given a diet deficient in vitamin A had significantly decreased activity levels of the male specific hepatic CYP isoforms 2C11 and 3A2, as well as suppressed levels of CYP2C11 protein expression.[14] In contrast, rats administered excessive amounts of vitamin A showed no changes in CYP2C11 or 3A2 activity as compared with controls. However, increased amounts of vitamin A resulted in increased CYP2A1 catalytic activity. Xu et al. reported that rats maintained on a zinc-deficient diet had a significant reduction in constitutive hepatic aminopyrine *N*-demethylase activity, but no significant alterations in benzphetamine *N*-demethylase activity.[15] Although zinc is critical in CYP gene expression and resulting protein stabilization,[16] altering zinc levels has a selective effect on CYP-mediated drug metabolism.

Dietary sodium intake has also been implicated in changing CYP activity in rats. Capdevila et al. reported that rats administered supplemental sodium chloride in their drinking water had a significant increase in total arachidonic acid metabolism by renal microsomes.[17] This increase was primarily attributed to a selective increase in microsomal epoxygenase activity to over four times the control values. In contrast, no changes in epoxygenase activity were found with hepatic microsomes, indicating that sodium chloride supplementation caused a tissue-specific increase in metabolic activity. However, it has been previously shown that a brief period of sodium depletion may decrease hepatic CYP2C11 and 3A2 metabolic activity.[18] Following a single dose of furosemide, to initiate sodium depletion, rats were fed a low-salt diet consisting of 0.05% sodium chloride. A marked decrease in hepatic microsomal activity was seen in these rats; however, as these rats were additionally administered olive oil as a vehicle agent, conclusive results regarding the relationship between salt depletion and hepatic drug metabolism could not be made. The purpose of the present study was to investigate the catalytic activity and protein expression of the specific rat hepatic CYP isoforms 2C11 and 3A2, as well as renal function, in the sodium-depleted and sodium-supplemented rat models.

MATERIALS AND METHODS

Materials

Furosemide was purchased from American Reagent Laboratories, Inc. (Shirley, NY). High-performance liquid chromatography (HPLC)-grade methanol and acetonitrile were purchased from Fisher Scientific (Pittsburgh, PA). Hydroxylated testosterone metabolites were purchased from Steraloids, Inc. (Wilton, NH). Acrylamide was purchased from National Diagnostics (Atlanta, GA). All other chemicals used in the study were purchased in

the highest grades available from Sigma Chemical Co. (St. Louis, MO).

Animals and Dosing

Thirty-six adult male Sprague Dawley rats were purchased from Charles River Breeders (Wilmington, MA). Rats were individually housed in plastic cages in a 12-hours light/dark cycle animal facility with controlled humidity and temperature. Rats were allowed free access to standard rodent chow (19% protein, 5% fat, 57% carbohydrate, and 0.23% sodium, Harlan Teklad, Indianapolis, IN) and deionized water ad libitum during a 1-week acclimation period. Following the acclimation period, rats were randomly divided into six equal groups. Twenty-four rats were continued on the standard rodent chow and 12 rats were started on a low-salt rice diet, containing identical percentages of protein, fat, and carbohydrate as the standard chow with the sodium level reduced to 0.01% (Harlan Teklad, Indianapolis, IN). Of the rats maintained on the standard rodent chow, six were given a single intraperitoneal dose of furosemide 4 mg/kg (STD+F) and six were given a single intraperitoneal dose of normal saline 1 mL/kg (STD). Of the 12 remaining rats given the standard diet, six received twice daily supplemental sodium via oral gavage of a sodium chloride solution at 500 mg/300 g body weight/day (3X) and six received supplemental sodium via oral gavage of a sodium chloride solution at a dose of 1.25 g/300 g body weight/day (6X). These supplemental doses of sodium approximate a three-fold and six-fold increase, respectively, in daily sodium intake as compared with the standard rodent chow alone. All rats not administered supplemental sodium were given an equal volume of water via oral gavage. Of the 12 rats started on the low-salt diet, six were given a single intraperitoneal dose of furosemide 4 mg/kg (Low+F) and six were given a single intraperitoneal dose of normal saline 1 mL/kg (Low). Table 1 lists the dosing groups in order of increasing daily sodium intake. Rats were maintained on their respective diets with or without sodium supplementation for 7 days. At the end of the 7 days, rats were placed in individual metabolic cages (Nalge, Rochester, NY), and urine was passively collected for 24 hours. After the urine collection period, rats were humanely sacrificed and blood samples were taken for serum electrolytes and creatinine determinations. In addition, livers were rapidly removed, rinsed in ice-cold normal saline, frozen in liquid nitrogen, and placed at –80°C until used for microsome preparation (within 1 week).

Microsome Isolation

Microsomes were isolated from frozen liver tissue by differential centrifugation as previously described.[19] Samples were maintained at 4°C during the microsome preparation. In brief, aliquots of liver tissue were homogenized in 3 mL of Tris chloride buffer, pH 7.4, containing 150 mM potassium chloride

Table 1. Adult male rats were maintained on either a low-sodium (Low) or standard (STD) rodent chow diet for 7 days. At the beginning of the study, rats were given either a single intraperitoneal injection of furosemide 4 mg/kg (F) or saline. Two additional rat groups were supplemented with oral dosing of a sodium chloride solution at 500 mg (3X) or 1.26 g (6X) per 300 mg body weight. Each group consists of six rats.

Group	Diet	Furosemide	Daily sodium supplements
Low+F	Low-salt	Yes	None
Low	Low-salt	No	None
STD+F	Standard	Yes	None
STD	Standard	No	None
3X	Standard	No	500 mg/300 g body weight
6X	Standard	No	1.25 g/300 g body weight

Adult male rats were maintained on either a low-salt (Low) or standard (STD) rodent chow diet for seven days. At the beginning of the study, rats were given either a single intraperitoneal injection of furosemide 4 mg/kg (F) or saline. Two additional rat groups were supplemented with oral dosing of a sodium chloride solution at 500 mg (3X) or 1.26 g (6X) per 300 g body weight. Each group consists of six rats.

and 1 mM EDTA, with a tissue homogenizer. Samples were then centrifuged at 9000 × g for 20 minutes at 4°C (GS 15R centrifuge, F0630 rotor; Beckman Instruments Inc., Palo Alto, CA). The supernatant was collected and centrifuged at 550,000 × g for 17 minutes at 4°C (TL100 centrifuge, TLA 100.4 rotor; Beckman Instruments Inc., Palo Alto, CA). The supernatant was discarded and the pellet was resuspended and washed in a sodium pyrophosphate buffer, pH 7.4, containing 1 mM EDTA with tissue homogenizer. The suspension was centrifuged again at 550,000 × g for 17 minutes at 4°C. The supernatant was discarded and the washed pellet was resuspended in a Tris chloride buffer, pH 7.4, containing 20% glycerol. Microsomes were stored at −80°C prior to analysis (within 2 weeks).

In Vitro Metabolism

For determination of in vitro metabolic activity the regio- and stereospecific hydroxylation of testosterone was measured as previously reported.[20] The production of 6β-hydroxytestosterone and 2α-hydroxytestosterone was measured as markers of CYP3A2 and 2C11 metabolic activity, respectively.[21,22] In brief, reaction mixtures contained 0.1 M potassium phosphate, pH 7.4, 0.2 mg microsomal protein, 250 mM testosterone in methanol, and an NADH regeneration system, consisting of 0.5 mM $NADP^+$, 10 mM glucose-6-phosphate, 10 mM magnesium chloride, and 5 U glucose-6-phosphate dehydrogenase. The total reaction volume was 1 mL. Mixtures were preincubated at 37°C for 3 minutes and reactions were initiated by the addition of glucose-6-phosphate dehydrogenase. Incubations proceeded for 15 minutes and were stopped by the addition of 5 mL of dichloromethane. The internal standard (3.6 nM 11-α-hydroxyprogesterone) was added to the samples while mixed on a vortex. The organic layer was removed and dried under a nitrogen stream. Dried extracts were dissolved in 200 μL methanol and stored at 4°C until analyzed (within 1 week).

Chromatography

Testosterone and metabolites were separated and quantified by HPLC. Aliquots (20 μL) of the extracts were injected into a Shimadzu HPLC system consisting of a model SIL-10A automatic injector, with dual model LC-10AS solvent pumps, and model SPD-10AV variable wavelength UV-VIS detector. Components were controlled by a model SCL-10A system controller, through CLASS-VP version 4.2 software (Shimadzu Corporation, Columbia, MD). Metabolites were resolved at 40°C, using a model CH-30 column heater with a model TC-50 controller (Eppendorf, Madison, WI) on a 150 mm × 4.6 mm C-18 column (Supelco, Bellefonte, PA), preceded by a 10 mm × 4.3 mm C-18 guard column (Upchurch Scientific, Oak Harbor, WA). A concave mobile phase gradient from 90% solvent A (methanol/water/acetonitrile, 39:60:1) to 85% solvent B (methanol/water/acetonitrile, 80:18:2) was delivered over 20 min at 1.5 mL/min. A 20 minutes washout of 100% solvent A preceded each analysis. Absorbance of hydroxylated metabolites was monitored at 238 nm. Testosterone metabolites were quantified by comparison of peak area ratios (metabolite/internal standard) with those generated with authentic standards. Rates were determined under conditions that were linear with microsomal protein concentration and incubation time.

Gel Electrophoresis and Immunoblot Analysis

Proteins were separated using sodium dodecyl sulfate polyacrylamide gel electrophoresis as described previously.[18] In brief, protein separation was achieved with gel electrophoresis using an 8% polyacrylamide separating gel followed by electrophoretic transfer of proteins to nitrocellulose sheets with an electrophoretic blotter (Idea Scientific Co., Corvallis, OR). For the detection of CYP3A2 immunoreactive proteins, nitrocellulose sheets were blocked with 5% nonfat dry milk (NFDM) in Tris-buffered saline (TBS, 10 mM Tris-chloride buffer, pH 7.4, containing 0.9% sodium chloride), blots were then incubated with a 1:2000 dilution of goat antirat CYP 3A2 antibody (GenTest, Woburn, MA) in 5% NFDM in TBS for 1 hour, and finally nitrocellulose sheets were incubated with a 1:2000 dilution of rabbit antigoat IgG (Sigma Chemical Co., St. Louis, MO) in 5% NFDM in TBS. The immunoreactive proteins CYP 2C11 were detected similarly. Blocked nitrocellulose sheets were incubated with a 1:2000 dilution of rabbit antirat 2C11 antibody (generously provided by Dr. Edward T. Morgan, Emory University, Atlanta, GA) followed by an incubation with a goat antirabbit horseradish peroxidase (1:2000 dilution) (Bio-Rad Laboratories, Hercules, CA) in 5% NFDM in TBS. Immune complexes were detected

by chemiluminescence with an electrogenerated chemiluminescence (ECL) detection kit as described by the manufacturer (Amersham, Arlington Heights, IL) using Kodak X-OMAT AR film (Eastman Kodak Company, Rochester, NY). Band density was measured using a flatbed scanner (LaCie, Beaverton, OR) and analyzed on a Power Macintosh 7200/90 computer using the public domain NIH Image program version 1.60 program (developed at the U.S. National Institutes of Health and available on the internet at http://rsb.info.nih.gov/nih-image/).

Additional Assays

Microsomal protein concentrations were determined by the method of Lowry et al.[23] using a commercially available assay kit (DC Protein Microassay; Bio-Rad, Hercules, CA). Urine and serum sodium and potassium concentrations were measured using a Jenway PFP7/C flame photometer (Cole-Parmer, Vernon Hills, IL). Urine and plasma creatinine concentrations were measured using a colorimetric assay kit (Sigma Chemical Co., St. Louis, MO). Creatinine clearance was determined by measuring urine creatinine divided by serum creatinine multiplied by urine flow rate. Parameters of renal function were estimated by standard methods.[24]

Statistical Analysis

Differences among rat groups were determined using one-way analysis of variance with Bonferroni-Dunn posthoc analysis using the standard (STD) group as a control (SuperANOVA; Abacus Concepts, Berkeley, CA). Data are presented as mean ± standard error. Differences were considered significant when the probability of chance explaining the results was reduced to less than 5% ($P < 0.05$, $\alpha = 0.05$).

RESULTS

Renal Function

The effect of sodium intake on markers of renal function is given in Fig. 1. Water intake increased 41% ($P = 0.002$) in the 6X group, as compared with STD controls. Although rats receiving the low-salt diet consumed less water, it was not statistically different from controls. Passive urine output over a 24-hour period was significantly greater in 6X rats ($P < 0.001$) and statistically lower in Low rats ($P = 0.043$), but only moderately lower in Low+F rats ($P = 0.071$), as compared with the STD group. Analysis of serum creatinine revealed no statistical differences amongst the differing treatment groups. Urine creatinine levels were decreased in the groups with 3X and 6X salt supplementation, as compared with STD controls; however, these differences were not found to be statistically significant. Creatinine clearance was reduced by 45% in Low+F rats ($P = 0.015$) and 44% in Low rats ($P = 0.013$), as compared with controls. As expected, the fractional excretion of sodium in the urine reflected the respective daily sodium chloride intake. Rats in the 3X and 6X groups had urinary sodium excretion rates that were 225% ($P < 0.001$) and 232% ($P < 0.001$) greater then the rates of control rats. Rats in the Low+F and Low treatment groups had rates that were reduced to 38% ($P = 0.039$) and 50% ($P = 0.078$) of the rates of STD controls.

Immunoblot Analysis

Hepatic microsomal CYP3A2 and 2C11 enzymes were measured using Western blot analysis. Figure 2 shows the relative densities of both CYP isoforms in hepatic microsomes. No statistical differences in CYP3A2 immunoreactive proteins were detected between groups. However, hepatic microsomal CYP2C11 was elevated by 37% ($P < 0.001$) in rats receiving the largest sodium supplements as compared with STD rats.

In Vitro Metabolism

The specific activity of CYP3A2 and 2C11 enzymes was measured by determining the in vitro microsomal production of 6β-hydroxytestosterone and 2α-hydroxytestosterone, respectively. The data are presented in Fig. 3. No significant differences were found in the production of 6β-hydroxytestosterone amongst treatment groups. Although rates were somewhat lower in rats receiving the low-salt diet, differences from STD controls did not reach statistical significance. In vitro production of 2α-hydroxytestosterone, a marker of CYP2C11, was significantly reduced in rats given additional sodium supplementation. Rats in the 3X group had a 37% ($P = 0.002$) reduction in 2α-hydroxytestosterone production

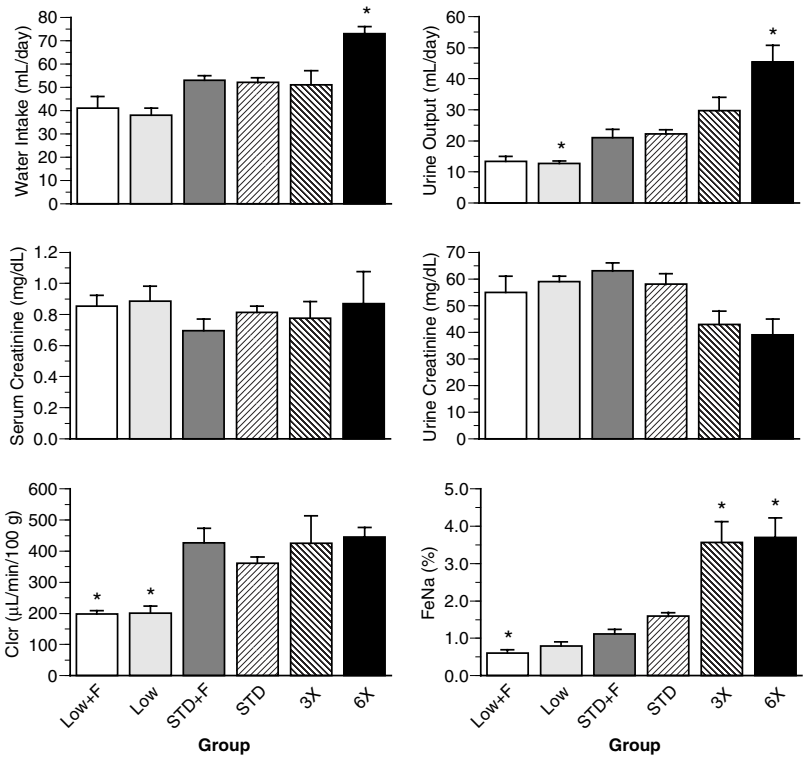

Figure 1. Markers of renal function and fluid balance in rats given varying amounts of dietary sodium intake. Treatment groups are the same as described in Table 1. Each group consisted of six rats. Data are represented as mean ± SE. **Abbreviations:* Clcr, creatinine clearance; FeNa, fractional excretion of sodium. *$P < 0.05$ as compared with STD group.

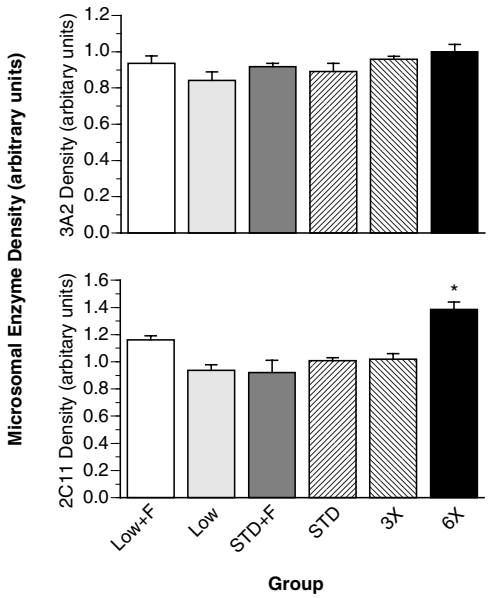

Figure 2. Hepatic microsomal protein levels in rats receiving various amounts of daily sodium intake. Treatment groups are the same as described in Table 1. Each group consisted of six rats. Data are represented as mean ± SE. *$P < 0.05$ as compared with STD group.

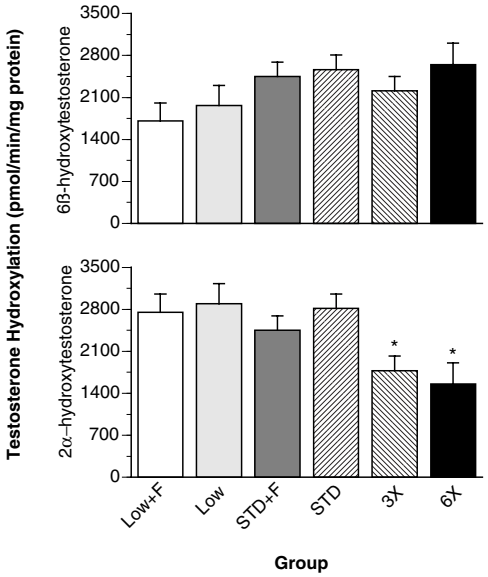

Figure 3. In vitro cytochrome CYP-dependent testosterone hydroxylation produced by hepatic microsomes from rats receiving varying amounts of dietary sodium. Treatment groups are the same as described in Table 1. Each group consisted of six rats. Data are represented as mean ± SE. *$P < 0.05$ as compared with STD group.

while rats in the 6X group had a 45% ($P = 0.006$) reduction.

DISCUSSION

Various factors are known to affect the metabolism of xenobiotics, including diet and nutrition. In order to determine if alterations in sodium affect cytochrome P450 enzymes, and thus possibly xenobiotic metabolism, we investigated the metabolic activity and protein expression of two major hepatic CYP isoforms, 3A2 and 2C11, in both the sodium-deprived and sodium-supplemented rat models.

The data from the present study indicate that sodium supplementation affects CYP2C11 protein expression and activity. We found that rats supplemented with three and six times the normal daily sodium intake had a significant reduction in the in vitro production of 2α-hydroxytestosterone, a maker of CYP2C11 metabolic activity. In addition, rats supplemented with six times the normal daily sodium intake had an increase in immunoreactive hepatic CYP2C11 protein levels. Despite the decrease in metabolic activity there was an increase in hepatic microsomal CYP2C11 immunoreactive protein expression, only seen with severe sodium loading. A possible explanation for this discrepancy is that the induced CYP2C11 protein has minimal or decreased catalytic activity and the induction is a result of protein stabilization and a lack of degradation. Our findings are similar to results contained in a report regarding hepatic CYP2C11 immunoreactive protein expression following modest salt loading.[17] In rats who were given sodium chloride (2.0–2.5% w/v) in drinking water over 10–12 days, there was no change in CYP2C11 proteins in the liver as compared with controls given no additional sodium chloride. It was only after severe sodium loading—six times the normal dietary intake—that we saw an increase in hepatic CYP 2C11 protein expression.

In contrast, both sodium restriction and supplementation resulted in no significant differences in CYP3A2 protein expression or activity. In contrast to our findings, Darbar et al. previously reported that normal volunteers maintained on a low sodium diet (10 mEq/day) had a greater absorption of the CYP3A substrate, quinidine, as compared with subjects receiving a high sodium diet (400 mEq/day).[25] The increased absorption however, was attributed to a decrease in the first pass effect. The quinidine concentration time profiles were different during the initial four hours following oral drug administration, while blood concentrations after intravenous administration were the same between groups, indicating a first pass effect. The effect of sodium chloride intake on intestinal CYP3A-mediated metabolism was not addressed but could be a contributing factor in the altered quinidine concentrations seen. Blood leaving the gastrointestinal tract makes its first pass through the liver, and if an alteration in sodium chloride results in intestinal CYP3A aberrations, systemic blood levels of CYP3A substrates could be modified.

Data from the present study show that sodium alterations result in changes in markers of renal function. Acute changes in sodium homeostasis are known to alter glomerular filtration rate and modify the untoward effects of nephrotoxic agents, such as cyclosporine and amphotericin B.[26] In fact, sodium depletion is commonly used to predispose rats to renal toxicity during chronic cyclosporine administration.[20] In addition, Gerkens and Branch reported that sodium-depleted dogs had a significant reduction in urine flow rate as compared with salt-loaded dogs.[27]

We found that in both rat groups maintained on a low-salt diet, daily urine volume and creatinine clearance were both reduced, as compared to rats maintained on a standard diet without sodium supplementation. In contrast, rats given twice daily sodium supplements had creatinine clearance values that were the same as rats receiving the standard diet alone. Since serum creatinine values were the same among all groups, creatinine clearance differences were due to 24-hour urine volume values among groups. Rats given the low-salt diet had significantly reduced urine output while rats receiving the largest sodium supplement had urine outputs over twice that of controls. Furthermore, rats appeared to maintain fluid balance, in that the relative water intake matched urine output for each group. The data show that acute sodium deprivation with a low-salt diet does alter renal function through changes in urine volume output similar to those seen previously in dogs.[27] However, this change is independent of the use of furosemide, which was used at the start of the study to initiate sodium depletion.

In summary, sodium aberrations in rats result in a decrease in estimated glomerular filtration rate due to a reduction in the urine flow rate. In addition, daily sodium intake has a selective effect on hepatic drug metabolism. Although we found no changes in CYP3A2 levels or catalytic activity with either sodium-deprived or sodium-loaded rats, sodium-loading induced hepatic CYP2C11 microsomal pro-

teins while suppressing CYP2C11 catalytic activity. Thus, experiments designed to use an altered sodium-diet animal model, to alter drug toxicity or effect, should use an appropriate control to account for altered renal function and specific CYP isoform function.

ACKNOWLEDGMENTS

This work was supported by a grant from the Burroughs Wellcome Fund and the American Foundation for Pharmaceutical Education through the AACP New Investigators Program for Pharmacy Faculty (LJB) and by grant GM60910 from the National Institutes of Health (LJB).

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