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Regulation and Characterization of the ATP-Binding Cassette Transporter-B1 in the Epididymis and Epididymal Spermatozoa of the Rat

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It has been reported that following administration, alkylphenols, such as octylphenol, reach the testis and epididymis but fail to accumulate in these tissues, suggesting the rapid expulsion of these chemicals by transporters. Specialized transporters that function to restrict compounds that enter target cells have been identified. ABCB1 is a member of the ATP-binding cassette family of proteins capable of transporting a broad range of drugs and xenobiotics out of tissues. The objective of this study was to characterize the expression profile and functional role of ABCB1a and ABCB1b along the different regions (initial segment, caput, corpus [CS], and cauda [CA]) of the adult rat epididymis. ABCB1a and ABCB1b transcripts were detected in all four regions of the epididymis. Immunolocalization revealed minimal ABCB1 staining in epithelial cells or spermatozoa of proximal regions of the epididymis; however, this progressively increased in the CS and CA epididymis. This expression gradient was confirmed by Western blot, suggesting that spermatozoa acquire ABCB1 during epididymal maturation. Multidrug resistance (MDR) assays revealed that rat epididymal cells and epididymal spermatozoa display an MDR phenotype that can be inhibited under control conditions. To assess whether or not the system was inducible by alkylphenols, cells from an immortalized epididymal cell line (RCE) were exposed to different concentrations of nonylphenol. Results revealed a significant induction of both ABCB1a and ABCB1b messenger RNA and ABCB1 protein in RCE cells. Our findings demonstrate a role for ABCB1 in protecting both epididymal principal cells and spermatozoa from xenobiotics.

Key Words: **ABC transporter; ABCB1; alkylphenol; epididymis; nonylphenol; spermatozoa.**

Spermatozoa are produced in the testis via spermatogenesis, but it is during epididymal transit that they acquire the ability to swim and fertilize. The epididymal epithelium consists of several specialized cell types, which exhibit region-specific localization and function that contribute to sperm maturation (Hermo *et al.*, 1992; Hinton and Palladino, 1995). Tight junctions between epithelial principal cells that line the lumen, as well as receptor-mediated transport of molecules to and from the lumen, create a specific luminal environment that is different from blood and that is necessary for sperm maturation (Cyr *et al.*, 2002, 2007; Wong *et al.*, 2002).

Several studies have shown that environmental toxicants can alter epididymal function and affect sperm maturation (Foster *et al.*, 2010; Klinefelter, 2002). Few studies, however, have examined the presence of efflux transporters in the epididymis and whether or not xenobiotics can be transported out of epididymal cells. The administration of phytoestrogens to the xenobiotic efflux transporter ABCG2 knockout mice resulted in elevated epididymal concentrations of phytoestrogens as compared with wild-type mice (Enokizono *et al.*, 2007), suggesting that efflux transporters may play a role in protecting the epididymis and maturing sperm from chemical insult.

Widely used industrial surfactants, such as alkylphenol ethoxylates, and their major degradation products, alkylphenols, have been proposed as male reproductive toxicants *in vivo* (Boockfor and Blake, 1997; Han *et al.*, 2004) and *in vitro* (Aravindakshan and Cyr, 2005). Recent *in vivo* studies, however, suggest that environmentally relevant doses of certain alkylphenols, such as 4-tert-octylphenol (OP), may be less toxic than originally reported (Gregory *et al.*, 2009). A possible reason for the lower reproductive toxicity of OP is that although it reaches the testis and epididymis, it is rapidly cleared from these tissues in adult rats (Hamelin *et al.*, 2009). This suggests that AP are either rapidly metabolized or transported out of the tissue.

The ATP-binding cassette (ABC) family of efflux transporters are plasma membrane proteins capable of transporting a wide array of chemically diverse substrates, including drugs, toxicants, lipids, and other endogenous substrates, across cellular membranes. Several members of this family, including ABCB1 (phospho-glycoprotein [P-glycoprotein] and multidrug resistance-1), ABCG2 (breast cancer resistance protein and mitoxantrone resistance protein), and ABCC1 (multidrug resistance–associated protein-1), were originally implicated in

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chemotherapeutic resistance but were later found to be constitutively expressed in normal tissues, serving a barrier or excretory function. These transporters actively regulate the disposition of numerous drugs and toxicants. ABCB1 was the first ABC efflux transporter to be identified (Juliano and Ling, 1976). It is constitutively expressed in the intestine, kidneys, liver, pancreas, adrenals, placenta, blood-brain, and blood-testis barriers (Staud *et al*., 2010; Thiebaut *et al*., 1987). ABCB1 is capable of translocating a wide range of primarily lipophilic and amphipathic substrates (Klaassen and Aleksunes, 2010; Mizutani *et al*., 2008; Staud *et al*., 2010). Whereas humans possess one *ABCB1* efflux gene, rodents have two ABCB1 efflux genes, *ABCB1a* and *ABCB1b*. Differences in the tissue distribution of ABCB1a/b have been reported, but the two genes are thought to collectively serve the same physiological function as human ABCB1 (Cui *et al*., 2009). ABCB1 has been localized to the vasculature of the testis and interstitial steroidogenic Leydig cells and was recently identified as an integrated component of the blood-testis barrier (Bart *et al*., 2004; Melaine *et al*., 2002; Su *et al*., 2009). ABCB1a knockout mice are viable and fertile under control conditions but display altered ABCB1a pharmacokinetic properties and are more susceptible to chemical injury (Schinkel *et al*., 1994). In ABCB1 knockout mice, digoxin entry into the testis was significantly increased compared with wild-type mice (Schinkel *et al*., 1995).

Given the established role of ABCB1 in the blood-testis and other blood-tissue barriers, we hypothesized a role for ABCB1 in epididymal barrier function and protection of sperm. The objectives of the present study were to characterize the expression profile and functionality of ABCB1 in the adult rat epididymis and epididymal spermatozoa, as well as to assess ABCB1 functionality and the ability of an alkylphenol, nonylphenol (NP), to induce ABCB1 expression in rat epididymal cells.

MATERIALS AND METHODS

Animals. Adult Sprague-Dawley rats were purchased from Charles River Canada (St Constant, Quebec, Canada) and acclimated for 7 days prior to surgery. Rats were maintained under a consistent photoperiod of 12 h light and 12 h darkness and received food and water *ad libitum*. All animal protocols used in this study were approved by the university animal care committee.

Rats were euthanized with CO_2 at the time of sampling. Epididymides were dissected and subdivided into four anatomical regions (initial segment [IS], caput [CT], corpus [CS], and cauda [CA] epididymidis). Epididymal tissues were either flash frozen in liquid nitrogen for RNA and protein analysis, prepared for immunolocalization studies, or used immediately for sperm extraction.

Real-time PCR. Total RNA was isolated from epididymal tissue and epididymal cells using the Illustra RNAspin Mini kit (GE Healthcare, Montreal, Quebec, Canada) according to manufacturer's instructions. Reverse transcription (RT)-PCR was performed using a Rotor-Gene RG3000 (Corbett Life Science, Mortlake, New South Wales, Australia). All reactions were performed in triplicate. Reverse transcription was done using 500 ng RNA, oligo $(dT)_{16}$ primers (R&D Systems Inc., Minneapolis, MN), and Moloney Murine Leukemia Virus reverse transcriptase (Sigma-Aldrich, Mississauga, Ontario, Canada). Real-time PCR reactions for ABCB1a, ABCB1b, ABCC1, ABCG2, and β-actin were done using primers listed in Table 1. PerfaCTa SYBR Green SuperMix (Quanta Biosciences Inc., Gaithersburg, MD) was used according to the manufacturer's protocol. PCR reactions contained 2 μl of RT reaction, 1× PerfeCTa SYBR SuperMix, and 0.9 μl (200mM final concentration) of both forward and reverse primers (total volume of 15 μl). Standard curves using appropriate complementary DNA were created for all genes examined and used to calculate relative expression levels. Relative messenger RNA (mRNA) levels of target genes of interest were normalized to β-actin. Statistical analyses were performed using one-way ANOVA ($p \leq 0.05$, ANOVA; Systat Software, Inc., Chicago, IL).

Cell culture and treatments. Rat epididymal epithelial RCE cells (Dufresne *et al*., 2005) were plated at an appropriate density in 6-, 24-, or 96-well cell-plus culture plates (Sarstedt Inc., Montreal, Quebec, Canada). Cells were cultured in Dulbecco modified Eagle medium/Ham nutrient mixture F12 (Sigma-Aldrich; containing 50 U/ml penicillin, 50 μg/ml streptomycin, 2mM L-glutamine, 10 μg/ml insulin, 10 μg/ml transferrin, 80 ng/ml hydrocortisone, 10 ng/ml epidermal growth factor, 10 ng/ml cyclic adenosine monophosphate, 5% fetal bovine serum, and 5nM testosterone) in a humidified incubator at 32°C with 5% CO_2 until they reached confluency.

To assess the induction of ABCB1, cells were cultured 90–95% confluent as previously described, medium was removed, and cells were washed three times in PBS. Fresh medium containing ethanol (0.1%, vehicle), NP (1–20μM; Pestanal, analytical standard, technical mixture, Sigma-Aldrich), or doxorubicin hydrochloride (50 or 500 ng/ml, DOX, Sigma-Aldrich), a known ABCB1 inducer (Mercier *et al*., 2003), was added to the cells. Cells were treated for 24 h under normal culture conditions. Culture medium was removed, and cells were washed three times in PBS. For protein extraction, radioimmunoprecipitation assay buffer (PBS, 1% Igepal CA-630, 0.5% sodium deoxycholate, 0.1% SDS, 10 μg/ml PMSF, 100μM sodium orthovanadate, and a protease inhibitor cocktail; Sigma-Aldrich) was added to each of the wells. Cells were scraped from the wells, collected with a pipette, and placed immediately on ice. After 30 min of shaking at 4°C, cells were placed at −80°C overnight. The next day, cells were centrifuged for 10 min at 10,000 × g. Aliquots of the supernatant were stored at −80°C. RNA extraction from RCE cells was done using the Illustra RNAspin Mini kit (GE Healthcare) according to the manufacturer's instructions.

Immunohistochemistry. Whole epididymides were excised, fixed by immersion in Bouin's fixative (Fisher Scientific, Ottawa, Ontario, Canada) for 24 h, dehydrated, embedded in paraffin, and sectioned (5 μm). For immunostaining, the tissue sections were rehydrated through a series of graded ethanol (100–50%), including 70% alcohol with 1% lithium carbonate for 5 min, to remove residual picric acid. The sections were also incubated in 300mM glycine for 5 min to block free aldehydes and washed in tris-buffered saline. Heat-induced epitope retrieval was performed in a microwave oven for 10 min in citrate buffer (1.8mM citric acid and 8.2mM sodium citrate). Immunolocalization was performed with the DAKO Catalyzed Signal Amplification System (DAKO, Carpenteria, CA). Tissue sections were incubated with an anti-ABCB1 mouse monoclonal antibody (1:50 dilution in DAKO blocking solution; Abcam, Cambridge, MA) in a humidified chamber for 1 h at room temperature. Preabsorption of the primary antibody with a 200-fold excess of a commercially synthesized (Invitrogen, Burlington, Ontario, Canada) ABCB1 immunizing peptide (VVEAQLDKAREGRTC; Georges *et al*., 1990) was used as a negative control. Epididymal sections were counterstained for 5 min with 0.1% methylene blue, dehydrated in ethanol, immersed in Histoclear (Fisher Scientific), and mounted in Permount (Fisher Scientific). Sections were examined under a Leica DMRE microscope.

Immunofluorescence. Epididymides were excised, frozen in optimal cutting temperature compound (Fisher Scientific) on dry ice, and stored at −80°C until sectioning. Tissue sections (5 μm) were cut using a cryostat and stored at −80°C until use. For immunostaining, the tissue sections were fixed in ice-cold methanol or ethanol for 20 min at −20°C and then washed in PBS.

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TABLE 1
Real-time PCR Primer Sequences and Amplification Conditions Used for Amplifying ABC Transporters and β-actin Target Genes

Gene/accession number	Primer sequence (5′-3′)	Cycling conditions	Product size (bp)	Reference
ABCB1a/NM_133401	F:GCATTCTGGTATGGGACTT R:GTCTTTTCGAGACGGGTA	95°C 5 s 55°C 15 s 72°C 15 s	282	Wang *et al.* (2009)
ABCB1b/NM_012623	F:CATTCTGCCGAGCGTTAC R:CCCGTGTAATAGTAGGCGTA	95°C 5 s 55°C 15 s 72°C 15 s	107	Wang *et al.* (2009)
ABCC1/NM_022281	F:CCCTGAAGAGCAGTGACCTC R:TAGGCTTGGTGGGATCTTTG	95°C 15 s 54°C 30 s 30°C 30 s	156	NCBI
ABCG2/NM_181381	F:TCTTCGCCTTCCAAAAGCTA R:AAACCAGTTGTGGGCTCATC	95°C 15 s 54°C 30 s 75°C 30 s	213	NCBI
β-Actin/NM_031144	F:TCTGTGTGGATTGGTGGCTCTA R:CTGCTTGCTGATCCACATCTG	95°C 15 s 54°C 30 s 75°C 30 s	69	OLIGO 6

Note. NCBI, National Center for Biotechnology Information.

Tissue sections were blocked for 1 h in PBS containing Triton X (0.3%, Sigma-Aldrich), bovine serum albumin (2%, Fisher), and normal goat serum (2%, Sigma-Aldrich) at room temperature. Tissue sections were incubated with an anti-ABCB1 mouse monoclonal antibody (1:20 dilution in blocking solution, Abcam) in a humidified chamber for 1 h at room temperature. Slides were washed in PBS and then incubated with Alexa 488 anti-mouse secondary antibody (1:500 dilution in blocking solution; Invitrogen, Carlsbad, CA) for 1 h at room temperature. The slides were then washed in PBS and counterstained with 4′-6-diamidino-2-phenylindole (Vector Laboratories, Burlington, Ontario, Canada). Preabsorption of the primary antibody with a 200-fold excess of a commercially synthesized (Invitrogen) ABCB1 immunizing peptide (VVEAQLDKAREGRTC; Georges *et al.*, 1990) was used as a negative control. Sections were examined under a Leica DMRE microscope.

Sperm extraction. Adult rat epididymides were excised and dissected into IS, CT, CS, and CA. Tissue sections were immediately placed in prewarmed PBS. For protein extraction, a protease inhibitor cocktail (Sigma-Aldrich) was added to the PBS solution. Epididymal tissue was placed in a petri dish on a slide warmer at 37°C and a 30-G needle used to perforate the tissue. The tissue was incubated for 10 min to allow sperm to exit the tissue. Sperm-containing fluid was collected with a pipette and placed in microcentrifuge tubes. Spermatozoa were pelleted at 200 g for 10 min and resuspended in lysis buffer for protein extraction or PBS for multidrug resistance (MDR) assays.

Western blot analysis. Frozen epididymal tissue was ground with a mortar and pestle in liquid nitrogen. Lysis buffer (1:3 wt/vol; 60mM Tris-HCl, pH 6.8, 2mM $CaCl_2$, 40mM octyl β-D-glucopyranoside, 1 μg/ml Pepstatin A; Sigma-Aldrich) supplemented with a protease inhibitor cocktail (Sigma-Aldrich) was added to tissue samples prior to homogenization with a Polytron (PowerGen 500, Fisher Scientific). Homogenized samples were transferred to microcentrifuge tubes and separated by centrifugation as previously described (DeBellefeuille *et al.*, 2003). Protein concentrations were determined using the Bradford Reagent (Sigma-Aldrich) according to the manufacturer's instructions.

Spermatozoa were resuspended in 100 μl of buffer containing 1.0% Igepal CA-630, 154mM NaCl, 0.4M Tris, and protease inhibitor cocktail (pH 8.0; Sigma-Aldrich; Morales *et al.*, 2008). Samples were placed on a shaker at 4°C for 30 min and then centrifuged at 10,000 × g for 10 min at 4°C. Aliquots of the supernatant, which contained the protein, were stored at −80°C for future analysis.

Proteins (50 μg) were diluted in 2× sample buffer and boiled at 95°C for 5 min prior to loading on a 6% polyacrylamide gel (5% stacking gel). Protein samples were separated by electrophoresis and transferred onto a nitrocellulose membrane (Bio-Rad Laboratories, Toronto, Ontario, Canada) at 400 mA for 1 h under cooling conditions. Protein transfer onto the nitrocellulose membrane was verified with Ponceau red stain (0.6% wt/vol, 1% acetic acid). Membranes were washed with double distilled H_2O and subsequently blocked in TBS Tween-20 (TBST) (0.25%) containing 5% powdered milk for 1 h at room temperature. Membranes were incubated overnight at 4°C with a monoclonal anti-ABCB1 primary antibody (1:100, Abcam) diluted in blocking solution. The next day, blots were washed three times in TBST and incubated with horseradish peroxidase–labeled goat anti-mouse secondary antibody (Santa Cruz, CA) for 1 h at room temperature. Membranes were washed with TBST and then incubated with Lumilight Western blotting substrate (Roche, Rockford, IL) according to the manufacturer's instructions. Protein levels were normalized to α-tubulin (rabbit polyclonal anti-α-tubulin, Abcam) and subsequently quantified by densitometry with a Fluor Image analyzer (Bio-Rad Laboratories). Statistical analyses were done using one-way ANOVA (statistical significance was set at $p \leq 0.05$; SigmaStat).

MDR assays. MDR assays were performed on epididymal RCE cells and epididymal sperm extracted from proximal (IS, CT, and CS) and distal (CA) regions of the adult rat epididymis using the Vybrant Multidrug Resistant Assay Kit (Invitrogen) according to manufacturer's instructions. Cells were incubated with calcein acetoxymethylester (calcein AM), an ABCB1 and ABCC1 substrate, in the presence or absence of specific inhibitors.

Cyclosporin A (CsA, ABCB1 inhibitor, Sigma-Aldrich), MK571 (ABCC1 inhibitor, Sigma-Aldrich), and verapamil (ABCB1 inhibitor, Invitrogen) were used as ABC inhibitors. Stock solutions of CsA (5 mg/ml), verapamil (10 mg/ml), and MK571 (5 mg/ml) were prepared in 100% ethanol and stored at −20°C. Inhibitors were serially diluted to 4× final concentrations in PBS on the day of the experiment. A stock solution of 2mM calcein in PBS was freshly prepared on the day of the experiment. Cell viability was determined using propidium iodide (PI, Sigma-Aldrich) according to procedures of Dengler *et al.*, (1995).

RCE cells were plated in 96-well plates and grown until 90–95% confluent. Medium was removed and the cells washed in PBS. Fresh PBS (50 μl) was added to each well, and additional PBS (50 μl), CsA (50 μl; 1, 15, or 30 μg/ml final), or MK571 (50 μl; 1, 15, or 30 μg/ml final) was added and incubated for 15 min at 37°C. Calcein stock solution (100 μl, 1mM final concentration) was then added to the cells and incubated for an additional 15 min at 37°C. The solution was removed from each of the wells and cells washed in PBS. Fresh medium (100 μl) was added to each well and calcein fluorescence measured using a fluorometer (485/530 nm). PI was then added to the culture medium (1 μg/ml final concentration), incubated for 15 min at 37°C, and fluorescence

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measured (530/590 nm). Plates were covered and frozen (in darkness) at −80°C for 24 h, and fluorescence was measured after thawing at room temperature. The relative number of viable cells was calculated by subtracting the initial PI measurement from PI measurement in cells following freezing and thawing (total cell number). Calcein fluorescence values were normalized relative total viable cell number. Statistical analyses were performed with one-way ANOVA ($p \leq 0.05$, SigmaStat).

Spermatozoa were extracted from epididymides of adult rats, as previously described, and resuspended in 1 ml PBS. Spermatozoa concentration was determined with a hemocytometer, and the suspension was diluted to 20 million cells per milliliter in PBS. Diluted sperm samples (250 μl) were added to microcentrifuge tubes and incubated with PBS (250 μl), MK571 (250 μl, 7.5 or 30 μg/ml final concentration), or verapamil (250 μl, 7.5 or 30 μg/ml final concentration). The tubes were gently mixed by inversion and incubated for 15 min at 37°C. At the end of the incubation, calcein stock solution (500 μl, 1mM final concentration) was added, gently mixed by inversion, and incubated for an additional 15 min at 37°C. Spermatozoa were washed three times in PBS and diluted to a final concentration of 10^6 cells per milliliter in PBS. Calcein fluorescence (geometric mean, 10,000 cellular events) was determined using a FACScan (Becton Dickinson, Oakville, Ontario, Canada). Sperm populations were gated during fluorescence-activated cell sorting analysis to exclude cells with altered physical characteristics.

RESULTS

ABCB1 Expression and Localization in the Adult Rat Epididymis

Relative mRNA levels of ABCB1a and ABCB1b were determined by real-time PCR. Transcripts for both orthologs were detected in all four regions of the adult rat epididymis (Figs. 1A and 1B). ABCB1a mRNA levels in distal regions of the epididymis (CA) tended to be higher, but differences were not statistically significant. ABCB1b mRNA levels were similar in all epididymal regions (Figs. 1A and 1B). ABCB1a transcripts were more dominantly expressed than ABCB1b in the epididymis.

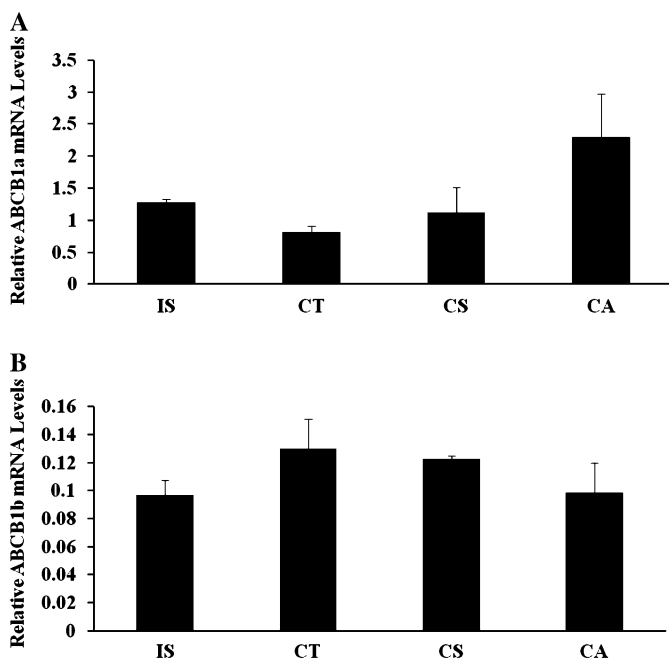

FIG. 1. Relative mRNA levels of ABCB1a (A) and ABCB1b (B) in the four segments of the adult rat epididymis (IS, CT, CS, and CA). Real-time PCR was done using gene-specific ABCB1a (A) and ABCB1b (B) primers and the data expressed relative to β-actin levels. Data are expressed as the mean ± SEM ($n = 3$).

ABCB1 immunohistochemistry using a mouse monoclonal antibody revealed a localization gradient of the protein along the epididymis. No immunostaining was detected in the epithelium or lumen of the IS (Figs. 2A and 2B). Minimal immunostaining was observed in the epithelial cells or spermatozoa in the CT (Fig. 2E) and progressively increased in distal CT, CS (Figs. 2G and 2H), and CA (Figs. 2J and 2K). A strong apical immunoreaction was observed in principal cells of the CS (Figs. 2G and 2H) and CA (Figs. 2J and 2K) regions. ABCB1 was also detected in a subset of clear cells, the principal endocytic cell in the epididymal epithelium, in distal regions (CA) of the epididymis (Fig. 2K). The appearance of ABCB1 in the epididymal epithelium coincided with a strong increase in spermatozoal immunostaining, indicating that spermatozoa acquire the protein during epididymal transit. ABCB1 was detected along the entire length of epididymal sperm in CS (Figs. 2G and 2H) and CA (Figs. 2J and 2K). An immunoreaction of varying intensity in interstitial blood vessels was also observed throughout the epididymis (Fig. 2). Preabsorption of the primary antibody with an immunizing peptide was used as a negative control (Fig. 2H).

Consistent with immunohistochemistry data, immunofluorescence revealed a similar gradient localization. No immunostaining was detected in the epithelium or lumen of the IS (Fig. 2C). Strong apical reaction was observed in the epithelium of distal CT, CS (Fig. 2I), and CA. ABCB1 was detected in individual clear cells in distal CT (Fig. 2F). A distinct immunopositive dot was observed on the midpiece of luminal spermatozoa under high magnification in CS and CA (Fig. 2L) regions. A variable immunostaining was also observed in interstitial blood vessels throughout the epididymis (Fig. 2). Preabsorption of the primary antibody with an immunizing peptide was used as a negative control (Fig. 2I).

Immunolocalization expression gradients were confirmed by Western blot analysis. Significantly more ABCB1 protein was detected in CS and CA regions of the adult rat epididymis as compared with the IS and CT (Figs. 3A and 3B). Similarly, sperm extracted from the CA region of the epididymis expressed significantly greater ABCB1 protein as compared with sperm extracted from IS and CT (Figs. 3C and 3D). Preabsorption of the primary antibody with excess peptide abolished all ABCB1 immunoreaction (data not shown).

ABCB1 Functionality in a Rat Epididymal Cell Line and Epididymal Sperm

To determine if ABCB1 is functional in the rat epididymis and epididymal spermatozoa, MDR assays were done using epididymal RCE cells (Fig. 4A) as well as isolated epididymal

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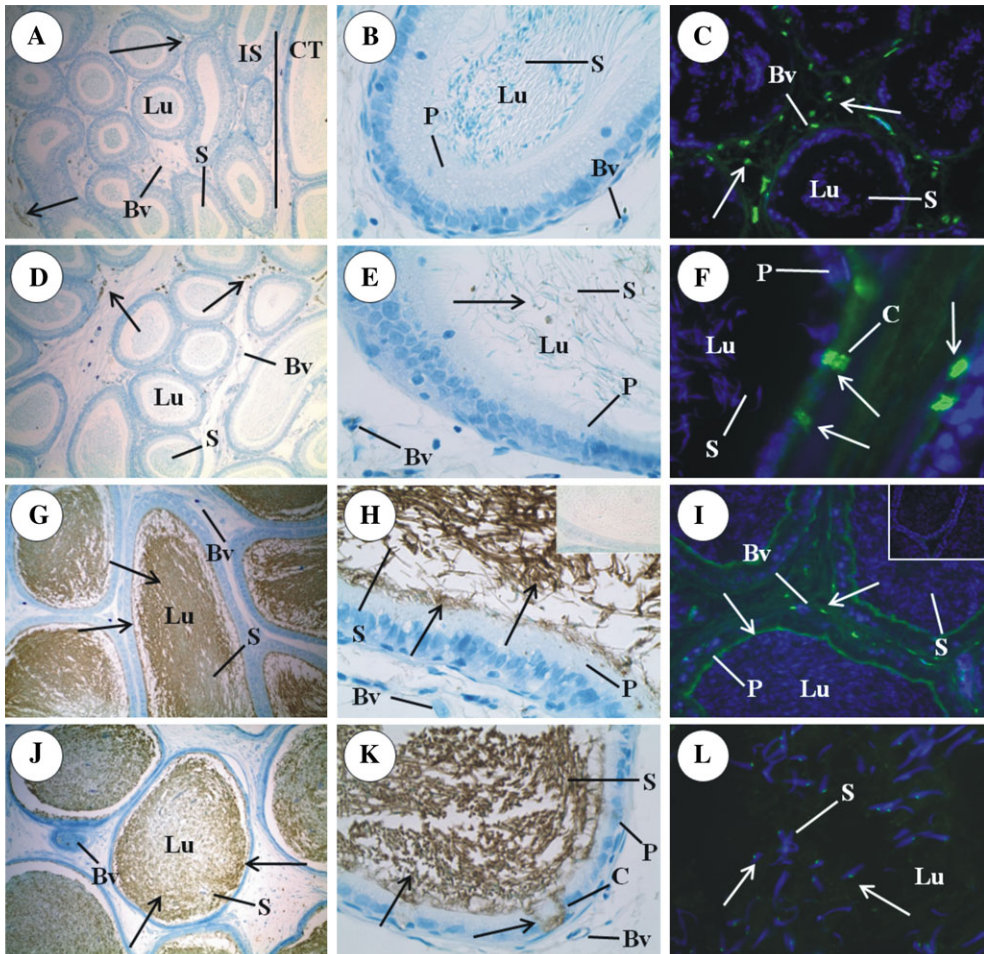

FIG. 2. Immunolocalization of ABCB1 in the four segments of the adult rat epididymis (IS, A–C; CT, D–F; CS, G–I; and CA, J–L). Arrows indicate positive immunostaining. Incubation of the ABCB1 antibody with excess specific immunizing peptide was used as a negative control (H and I, upper right panels). Bv, Blood vessel; C, clear cell; Lu, lumen; P, principal cell; S, spermatotozoa.

sperm from adult rats (Figs. 4B–D). Both RCE and epididymal sperm demonstrated an MDR phenotype that could be inhibited under control conditions. The low- and mid-dose CsA significantly increased calcein fluorescence in RCE cells (Fig. 4A), demonstrating ABCB1 activity. The highest dose of CsA did not produce any significant changes (Fig. 4A). The ABCC1 inhibitor, MK571, also significantly increased RCE cell calcein fluorescence in a dose-dependent manner (Fig. 4A), suggesting that ABCB1 is also active in RCE cells (Fig. 4A).

Epididymal sperm extracted from proximal (Figs. 4B–D) and distal (Fig. 4C) regions of the adult epididymis also displayed an inhibitable MDR phenotype. A dose-dependent increase in calcein florescence was observed in both proximal (Figs. 4B and 4C) and distal (Fig. 4D) sperm treated with an ABCB1 inhibitor, verapamil. A representative rightward shift in the calcein fluorescence peak was observed in verapamil-treated sperm as compared with untreated cells (Fig. 4B). In contrast, sperm from the CA incubated with MK571 demonstrated a dose-dependent decrease in calcein fluorescence (Fig. 4D).

Induction of ABCB1 mRNA and Protein in a Rat Epididymal Cell Line (RCE)

To determine if ABCB1 was inducible in the epididymal epithelium, relative mRNA and protein levels were measured in RCE cells following a 24 h exposure to varying concentrations of the known ABCB1 inducer, DOX, and to NP (Fig. 5). There was a tendency for ABCB1a mRNA to be increased by the highest DOX treatment (500 ng/ml), but this was not statistically significant (Fig. 5A). A greater than sixfold increase in ABCB1b mRNA was observed in DOX (500 ng/ml)-treated cells (Fig. 5C). Exposure to 10 and 20μM NP (Fig. 5B) resulted in two- and fourfold increases in ABCB1a mRNA levels. ABCB1b mRNA levels were also significantly increased in cells exposed to the highest NP concentration (20μM, Fig. 5D). There were no significant changes in ABCC1 or ABCG2 mRNA levels following DOX or NP treatment (data not shown).

Consistent with the mRNA data, a significant upregulation of ABCB1 protein was observed at the highest dose of DOX

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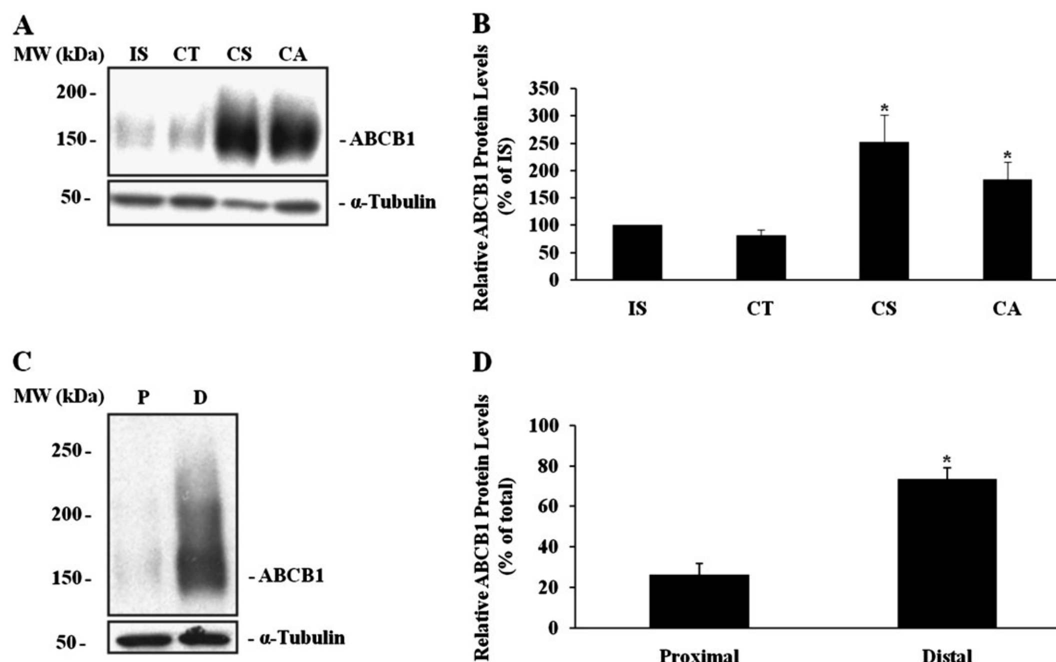

FIG. 3. Western blot analysis of ABCB1 protein expression in the four segments (IS, CT, CS, and CA) of the adult epididymis (A) and epididymal spermatozoa extracted from proximal (P, IS/CT) and distal (D, CA) regions (C) of the adult rat epididymis. Protein loading was standardized using α-tubulin as a protein loading control. Data are expressed as the mean percentage of IS ± SEM (B) or mean percentage of total ± SEM (D) of three different animals ($n = 3$). Asterisks represent a significant difference from IS (B) or proximal sperm (D) ($p < 0.05$, ANOVA). Incubation of the ABCB1 antibody with excess specific immunizing peptide abolished all ABCB1 immunoreaction (data not shown).

(Fig. 6). NP (10 and 20μM) increased ABCB1 protein expression in a dose-dependent manner (Fig. 6). Preabsorption with excess peptide abolished all ABCB1 staining at the expected molecular weight (data not shown).

Cell death varied in-between 2.1 and 10.7% in a dose-dependent manner for both DOX- and NP-treated cells (data not shown).

DISCUSSION

It has recently been reported that oral or intravenous administration of OP resulted in elevated OP levels in male reproductive tissues, which were rapidly cleared from the testis and the epididymis (Gregory *et al.*, 2009; Hamelin *et al.*, 2009). We hypothesized that cellular transporters such as ABCB1, a broad specificity xenobiotic efflux transporter, could play an important role in epididymal barrier function and spermatozoal protection and that it may be involved in the expulsion of alkylphenols from these tissues.

Our results demonstrate that ABCB1 is constitutively expressed and functional in both epididymal epithelial cells and in epididymal spermatozoa. ABCB1a mRNA levels were significantly higher in all regions of the epididymis relative to ABCB1b levels. Tissue-specific and gender-divergent expression of ABCB1 orthologs has been reported previously in mice (Cui *et al.*, 2009). Our results suggest that ABCB1a is the dominant ABCB1 in the adult rat epididymis but that both forms may play a role in epididymal barrier function.

As in other physiological systems, ABCB1 was detected in the capillary blood vessels of the epididymis. The presence of ABCB1 on the apical side of endothelial cells has been demonstrated previously to limit the penetration of circulating xenobiotics into sensitive organs and tissues (Schinkel *et al.*, 1994, 1995). ABCB1 immunostaining was also observed on the apical side of the epididymal epithelium in CS and CA epididymidis, suggesting a role in epididymal tissue defense. Although no directional efflux assays were conducted in the present study, directional studies in other tissues have suggested that ABCB1 pumps in a basal to apical direction, therefore, toward the epididymal lumen. Compounds capable of circumventing the endothelial barrier may therefore be actively transported out of the epididymal epithelium and into the lumen. Although not usually considered as such, the epididymis, like the kidney, liver, and mammary gland ducts, can act as an excretory tissue. Toxic metabolites, including heavy metals, pesticides, and industrial compounds, have been previously detected in semen (Stachel *et al.*, 1989). Paradoxically, vulnerable transiting and maturing sperm are also present in the epididymal lumen. Although advantageous for the epididymal epithelium, directional efflux toward the epididymal lumen may inadvertently expose sperm to toxic substances. Because spermatozoa are transcriptionally incompetent cells, they may be more susceptible to chemical insult and require specific defense mechanisms.

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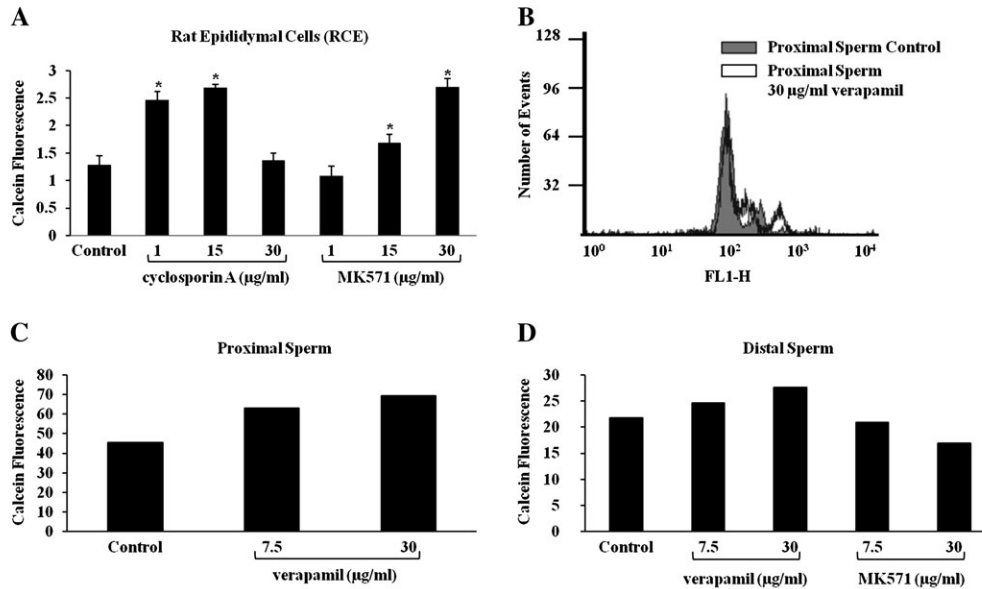

FIG. 4. MDR assay using rat epididymal RCE cells (A) and epididymal sperm (B, C, and D) extracted from the proximal (IS/CT/CS) and distal (CA) regions of the adult rat epididymis. RCE data are represented as mean calcein fluorescence (485/530 nm) normalized to total cell number as determined by PI ($n = 3$) (A). Asterisks represent a significant difference from control and vehicle treatment groups (ANOVA, $p < 0.05$). Flow cytometric analysis of proximal and distal epididymal sperm, represented as geometric mean fluorescence of 10,000 events, treated with verapamil (7.5 and 30 µg/ml), MK571 (7.5 and 30 µg/ml), or PBS (controls) was done. A representative flow cytometric analysis is shown (B). Calcein fluorescence in proximal (C) and distal (D) epididymal sperm was evaluated. Experiment was repeated three times with consistent results ($n = 3$). Data from one experiment are shown.

A clear cell-specific localization of ABCB1 was observed in epithelium of the adult rat epididymis. Clear cells are the principle endocytic cells in the epididymal epithelium (Hermo *et al.*, 1988). It is possible that the ABCB1 immunoreaction of these cells resulted from the endocytosis of ABCB1-positive spermatozoal components and cellular debris from the epididymal lumen. Clear cells have also, however, been demonstrated to express several xenobiotic-metabolizing enzymes, including cytochrome P450 (CYP) 1A1 and CYP2E1 (Forkert *et al.*, 2002; Roman *et al.*, 1998). Interestingly, there is

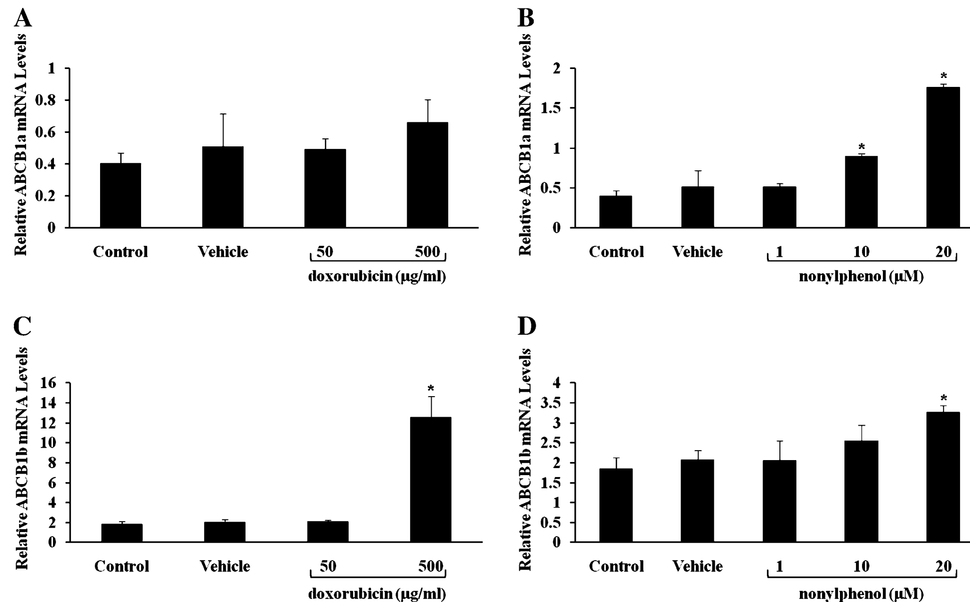

FIG. 5. Relative mRNA expression levels of ABCB1a (A and B) and ABCB1b (C and D) in RCE cells following 24 h of exposure to medium alone, vehicle (0.1% ethanol), NP (1, 10, and 20µM), or DOX (50 and 500 ng/ml). ABCB1a and ABC1b mRNA levels were determined by real-time PCR and normalized to β-actin mRNA levels ($n = 3$). Data are expressed as the mean ± SEM. Asterisks indicate a significant difference from control and vehicle treatment groups (ANOVA, $p < 0.05$).

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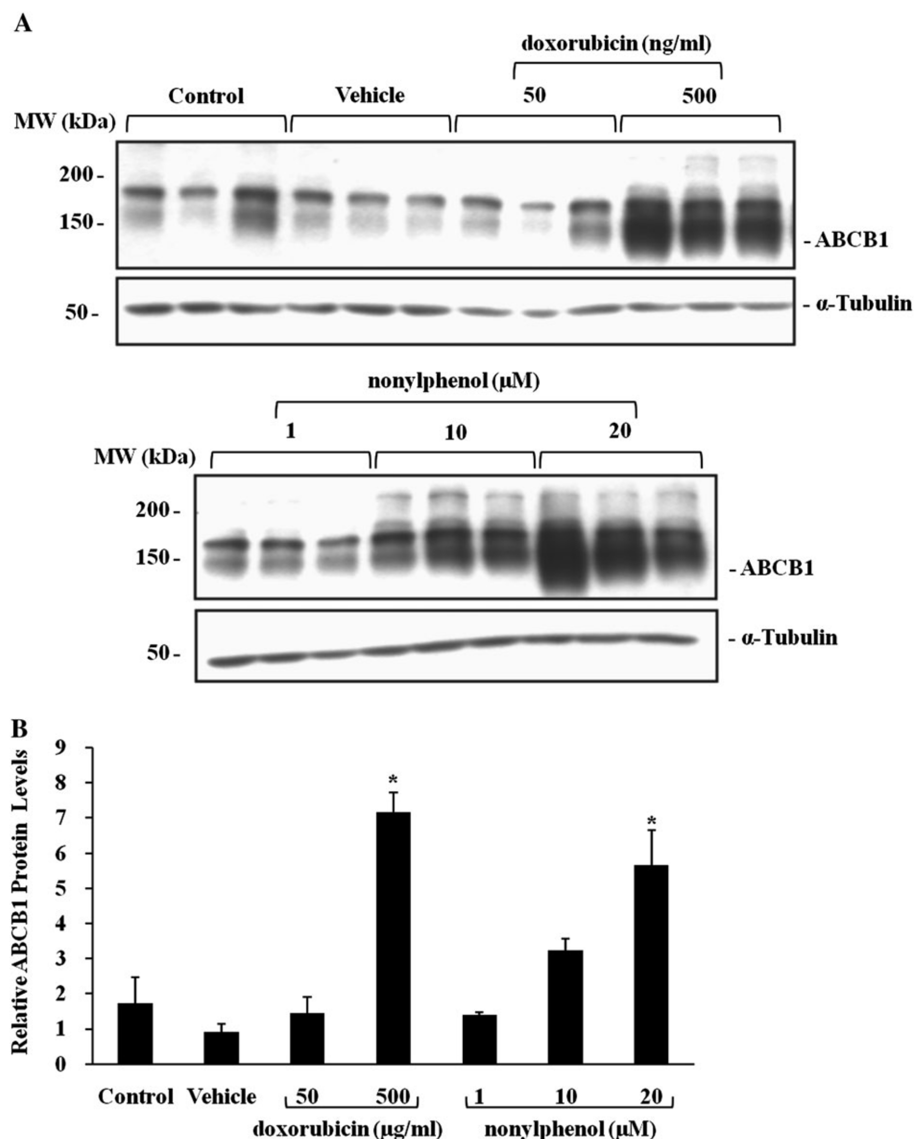

FIG. 6. Western blot analysis of ABCB1 protein expression in RCE cells following 24 h of exposure to medium alone, vehicle (0.1% ethanol), NP (1, 10, and 20μM), or DOX (50 and 500 ng/ml). Protein loading was standardized using α-tubulin as a protein loading control. Incubation of the ABCB1 antibody with an excess of specific immunizing peptide abolished all ABCB1 staining (data not shown).

considerable overlap in substrate specificity between ABCB1 and certain CYP450s (Staud *et al.*, 2010). Coordinated regulation of ABCB1 and CYP450s has also been reported in other tissue systems (Xu *et al.*, 2005). An alternative hypothesis, therefore, may be that epididymal clear cells act in concert with drug-metabolizing enzymes and xenobiotic efflux transporters to uptake, metabolize, and subsequently excrete luminal toxicants in more soluble forms.

Our findings demonstrated unique ABCB1 protein expression gradients in both the epididymal epithelium and in epididymal spermatozoa. Immunolocalization of ABCB1 was characterized by two different detection methods and further verified by Western blot. Using immunofluorescence, ABCB1 was localized to the midpiece of the spermatozoa. In immunohistochemistry, the localization encompassed the entire sperm, most likely because of the amplification of the ABCB1 signal by the DAKO amplification kit.

The expression gradient in epididymal sperm was particularly interesting, given that spermatozoa are transcriptionally incompetent. Any transcripts that they possess must be present in the testis or acquired from the epithelium during epididymal transit. Transporters of carnitine, an important biological molecule for sperm maturation and acquisition of motility, have been previously localized to epididymal sperm in a similar gradient pattern (Kobayashi *et al.*, 2007). Interestingly, several proteins, including both surface and glycosylphosphatidylinositol-anchored

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proteins, have been demonstrated previously to be transferred from the epithelium to sperm (Sullivan *et al.*, 2007). An alternative possibility is that a nonfunctional form of ABCB1 is already present in testicular sperm but only unmasked to a functional form during epididymal transit: ABCB1 is a P-glycoprotein with several potential posttranslational modification sites (Juliano and Ling, 1976). This notion is supported by the report of Oko *et al.* (1993) who identified the presence of a functional Golgi in the cytoplasmic droplet of epididymal spermatozoa. The Golgi may, e.g., increase the glycosylation of ABCB1, allowing the protein to be recognized by the antibody. In any case, the presence of ABCB1 represents a unique mechanism by which spermatozoa may defend against certain classes of environmental toxicants.

Both RCE and epididymal sperm demonstrated ABCB1 activity that could be inhibited under control conditions. Surprisingly, RCE cells incubated with the highest dose of CsA did not display any significant changes in calcein fluorescence. This may be because of a negative effect on endogenous esterase activity required for calcein AM conversion into fluorescent calcein. Although viability was accounted for by normalizing to total viable cell number, there is no measure for endogenous esterase activity in this assay. Further investigation is necessary to elucidate this phenomenon. ABCB1 activity was demonstrated in both sperm taken from proximal and distal regions of the epididymis. Proximal sperm populations included both sperm taken from IS and CT. Because ABCB1 expression begins in distal CT, the proximal sperm population is likely to contain both ABCB1-positive and ABCB1-negative cells. Consistent with the ABCB1 expression data, control calcein fluorescence levels were higher in proximal sperm compared with distal, suggesting lower ABCB1 activity.

Constitutive expression and function of ABCB1 indicates that in addition to a defense function, this protein has a normal physiological role in epididymal function and possibly sperm maturation. An association between male infertility and an ABCB1 polymorphism was demonstrated recently, suggesting a role in the maintenance of male reproductive potential (Drozdzik *et al.*, 2009). Whether or not the role of ABCB1 in infertility is because of a direct physiological function or a loss of defense mechanisms remains to be clearly established. ABC transporters have also been implicated in sterol and phospholipid transport to apolipoproteins and high-density lipoprotein in spermatozoa (Morales *et al.*, 2008). Endogenous sterols are known ABCB1 substrates and are important in the regulation of sperm capacitation and acrosome reaction (de Lamirande *et al.*, 1997; Garrigues *et al.*, 2002). Interestingly, the expression gradient in both the epididymal epithelium and epididymal spermatozoa coincides with changes in epithelial, luminal, and sperm plasma membrane sterol composition, suggesting a role for ABCB1.

Induction of ABCB1 mRNA and protein by DOX and NP, in dose-dependent manner, was demonstrated in RCE cells. As reported previously in mice, ABCB1b mRNA, but not ABCB1a, was significantly induced by DOX exposure (Mercier *et al.*, 2003). Both ABCB1a and ABCB1b mRNA were significantly induced by NP exposure, indicating the ability of alkylphenolic compounds to regulate both for rodent forms of ABCB1. The fact that ABCB1 is inducible indicates the presence of a dynamic defense system that can respond to chemical insult. Although NPEs have been shown to modulate ABCB1-mediated efflux (Doo *et al.*, 2005), to the best of our knowledge, this is the first study to demonstrate an induction of ABCB1 by an AP. These results may explain why OP is rapidly cleared from the epididymis of treated animals (Hamelin *et al.*, 2009).

MDR functional assays suggest that ABCC1 may also play a role in epididymal tissue defense. Additionally, although no induction was observed following DOX and NP treatment, mRNA transcripts for both ABCC1 and ABCG2 were detected in the RCE cells (data not shown). These findings suggest that several other members of the ABC transporters family may be implicated in epididymal function.

In summary, our results indicate that ABCB1 represents a toxicokinetic regulator of AP and other xenobiotics in the adult rat epididymis. The unique expression profile, localization, and induction of ABCB1 suggest a role in epididymal barrier function. Furthermore, the presence of ABCB1 in epididymal spermatozoa represents a unique defense mechanism for vulnerable spermatozoa against chemical insult.

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