Mitochondrial Dysfunction Occurs before Transport or Tight Junction Deficits in Biliary Epithelial Cells Exposed to Bile from Methylenedianiline-Treated Rats

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Methylenedianiline (DAPM) rapidly injures biliary epithelial cells (BEC) in vivo. Prior to evident BEC injury, biliary glucose and inorganic phosphate appreciably rise, which could stem from loosened tight junctions (TJ). Concurrently, ultrastructural abnormalities in BEC mitochondria of DAPM-treated animals are observed, suggesting other impairments. Our objective was to develop an in vitro BEC model to assess the time course of impairments in TJ integrity, glucose uptake, and mitochondrial function following DAPM exposure. We exposed monolayers of primary, polarized rat BEC to bile collected from rats prior to (Basal Bile) or after oral treatment (DAPM-Bile) with 50 mg DAPM/kg. DAPM-Bile collected during 0–60 min (1st Hr) and during 61–120 min (2nd Hr) after treatment was pooled from four to six rats. When monolayers were exposed to 1st Hr DAPM-Bile for 120 min, metabolic activity (XTT assay) decreased ~75%, and transepithelial resistance decreased ~16% in agreement with an ~65% increase in leakage of a glucose analog, methyl-α-D-glucopyranoside (AMG), from apical to basolateral media. By 60 min, AMG uptake was decreased ~40%. Mitochondrial function was very rapidly compromised, with ~120% increases in the green-to-red fluorescence ratio of JC-1 (mitochondrial membrane potential dye) at 15 min and ~55% decreases in ATP levels at 30 min. This sequence of events indicates that DAPM impairs BEC mitochondria prior to impairments in glucose uptake or TJ integrity. Thus, our in vitro primary rat BEC bile exposure model mimics in vivo observations and yields basic information about the time course of events that occur during DAPM-induced injury.

Key Words: methylenedianiline; biliary epithelial cells; bile; mitochondria; tight junctions; glucose transport.

Biliary epithelial cells (BEC) or cholangiocytes are known targets of injury by various insults such as drugs, ischemia/reperfusion, autoimmunity, and infection (Strazzaboso et al., 2000), which could lead to the development of chronic cholangiopathies. Early events in the BEC injury produced by these various insults are poorly understood. Recent establishment of BEC isolation and culture techniques has provided a new approach to address these unknowns. We selected 4,4′-diaminodiphenyl-methane (DAPM) as a representative insult for an in vitro model of injury to cultured BEC because of the human health relevance of this cholangiodestructive chemical. DAPM is employed in the manufacture of polyurethanes, which are made into tubing and films used in dialysis, plasma separation, intra-aortic balloons, and vascular grafts (Shintani and Nakamura, 1989). Toxicity of DAPM (also known as methylenedianiline) was first described in 1965, when residents of Epping, England developed upper abdominal pain, jaundice, malaise, and rash following ingestion of DAPM-contaminated bread; biopsies indicated cholangitis, cholestasis, and portal inflammation and edema (Kopelman et al., 1966). Other cases with similar patterns of liver injury have been linked to accidental or occupational exposure to DAPM (Bastien, 1984; Tillmann et al., 1997).

Time-course studies of rats given 100–250 mg DAPM/kg demonstrated that BEC necrosis is the first evident histopathologic change, followed by portal tract edema, fibrin exudation, endothelial and periportal hepatocellular necrosis, marked inflammatory infiltrate, cholestasis, and elevations in serum markers of hepatic injury (Ballie et al., 1993; Kanz et al., 1992). Lower doses of DAPM (25–50 mg/kg) cause a striking rise in biliary inorganic phosphate (Pi) and glucose within 3 h, without major effects on bile flow or other bile constituents (Dugas et al., 2001; Kanz et al., 1998). By 6 h, BEC become necrotic and slough from bile ducts while serum indicators of hepatotoxicity are only mildly elevated. Concurrent with the early elevations of biliary Pi and glucose, mitochondria in BEC display abnormal cristae and translucent matrices (Kanz et al., 1998). The mechanism(s) by which DAPM treatment leads to BEC injury and alterations in biliary constituents is unknown.

Elevated biliary Pi is usually considered a marker of alterations in the paracellular pathway of bile formation. Numerous cholestasis-inducing toxicants such as carmustine (Krell et al., 1991), α-naphthylisothiocyanate (ANIT) (Krell et al., 1982), and estradiol valerate (Jaeschke et al., 1987) elevate biliary Pi levels. Glucose is proposed to enter bile via tight junctions (TJ)
(Handler et al., 1994), but to be maintained at low levels in bile by Na\(^+\)-dependent glucose transporters on BEC (Lazaridis et al., 1997; Lira et al., 1992). Thus, paracellular “leakiness” could explain the DAPM-induced elevations in both glucose and P\(_i\). Yet DAPM only marginally increased \(^3\)H-inulin movement from plasma to bile in rats (Santa Cruz, unpublished observations) and, thus, appears to minimally alter paracellular permeability. Alternatively, the ultrastructural alterations in BEC mitochondria that occur rapidly after DAPM treatment (Kanz et al., 1998) are relevant to the elevations of biliary P\(_i\) and glucose, because the functional integrity of TJ (Denker and Nigam, 1998) and the absorption of biliary glucose via the Na\(^+\)-dependent glucose transporter, SGLT1 (Lazaridis et al., 1997), are dependent upon ATP.

In order to assess the temporal relationship of these impaired functions, we developed a novel in vitro model system to expose BEC to bile from control or DAPM-treated rats. Bile was selected as the vehicle for delivery of injurious entities based on prior animal-to-animal bile transfer studies which indicated that the proximate toxicant(s) of DAPM is excreted into bile (Kanz et al., 1995). Identity of the DAPM proximate toxicant(s) is unknown because bile contains a large number of metabolites, of which less than 20\% have been characterized (T. R. Dugas, unpublished observations). Our objective was to determine the time course of DAPM-Bile effects on the interrelated endpoints of (1) \(^{14}\)C-DAPM uptake into BEC, (2) mitochondrial membrane potential (MMP) and ATP levels, (3) Na\(^+\)-dependent uptake of a glucose analog, (4) leakage of this glucose analog through BEC tight junctions, (5) BEC monolayer transepithelial resistance (TER), and (6) cytotoxicity. Our hypothesis was that primary BEC monolayers would mimic the critical events in DAPM-induced biliary injury in vivo. These studies demonstrate that the in vitro observations of DAPM injury are reproduced in our in vitro BEC/bile exposure model and that impairments in BEC cellular function can be temporally distinguished. Furthermore, our results authenticate this in vitro system as a novel model for mechanistic-based studies of chemical/drug-induced cholangiopathies.

**MATERIALS AND METHODS**

Radiochemicals. \(^{14}\)C-DAPM (specific activity 6.8 mCi/mmol, \(>99\%\) pure) and \(^{14}\)C-methyl-\(\alpha\)-D-glucopyranoside (\(^{14}\)C-AMG, specific activity 300 mCi/mmol, \(>99\%\) pure) were obtained from American Radiolabeled Chemicals, Inc. (St. Louis, MO).

Animals. Male Sprague-Dawley rats (\(\sim 12\) weeks old; \(\sim 350\) g) from Harlan (Indianapolis, IN) were housed in wire-bottom cages in a 12-h light/dark cycle, temperature (range, 64–79°F) and humidity (range, 40–65%)–controlled animal room for 1 week prior to BEC isolation or bile collection. Rats were fed chow and water ad libitum. The Institutional Animal Care and Use Committee of the University of Texas Medical Branch approved all animal care and experimental procedures.

Characterization of rat BEC and in vitro experiments. Rat BEC were isolated and maintained using the methods of Yang et al. (1993) and Okamoto et al. (1995). BEC cultured by these methods are reported to retain adult antigenic markers through passage 40, but begin to display some phenotypic changes (e.g., heterogeneity of size) by passage 19. Thus, all experiments were conducted on BEC below passage 20. Polarization of BEC monolayers was determined by electron microscopy (see Fig. 7). Characterization of BEC throughout passages was accomplished by immunohistochemical staining for BEC specific markers (OC-2 antigen and cytokeratin 19) (Yang et al., 1993) (Fig. 1) and by histochemical staining for \(\gamma\)-glutamyl transpeptidase (Basachi et al., 1981) (data not shown). Cytokeratin-19 antibody was purchased from Immunotech (Marseilles, France).

Cells were suspended in serum-free defined media (Yang et al., 1993), seeded at a density of \(5.0 \times 10^5\) cells/ml on collagen-coated cultureware (1 part collagen stock: 4 parts sterile distilled water), and grown to confluence at \(37^\circ\) C in 5\% CO\(_2\). Collagen stock was made by dissolving 1 g of rat tail collagen in 300 ml 0.1% acetic acid, stirring for 48 h at 4\° C, and filtering through gauze. Monolayers were exposed for 15 min to 2 h on their apical surfaces only to undiluted, pooled (four to six rats) bile samples previously collected and frozen as described below or with solutes dissolved in iso-osmotic NaCl (iso-NaCl) consisting of 140 mM NaCl, 5 mM KCl, 1.3 mM CaCl\(_2\)-2H\(_2\)O, 0.5 MgCl\(_2\)-6H\(_2\)O, 10 mM HEPES, and 5 mM D-glucose adjusted to a pH of 7.4 with NaOH.

**Bile collection.** On days of bile collection, two rats were anesthetized with pentobarbital (50 mg/kg), and biliary and duodenal cannulas were implanted by standard surgical procedures (Kanz et al., 1992). Surgery duration was 30–45 min. Taurocholate (36 mM) was infused at \(\sim 3.2\) ml/h/kg rat into the duodenal cannula to maintain bile flow. Anesthesia was maintained by ip infusion of pentobarbital (2.5 mg/ml in saline) via a PE-50 cannula. Rats were equilibrated for \(\sim 20\) min on a heating pad until \(37^\circ\) C, and basal bile was collected on ice for 1 h. Rats were then gavaged (2 ml/kg) with 50 mg DAPM/kg dissolved in 35\% ethanol at 35–40\° C or vehicle (35\% ethanol), and bile was collected on ice for another 2 h. To obtain bile containing radioactive \(^{14}\)C-DAPM metabolites, rats were treated with 50 mg DAPM/kg that included \(^{14}\)C-DAPM (\(\sim 8.5\) μCi/rat).

Bile samples collected from two rats during the basal period, the first hour, and the second hour after DAPM administration [Basal Bile, DAPM-Bile (1st Hr), DAPM-Bile (2nd Hr), respectively] were pooled and stored in 200-μl aliquots at \(-80^\circ\) C. Pooled bile samples were assayed for glucose levels by Sigma kit 20. Bile acids (cholic, deoxycholic, chenodeoxycholic, hyocholic, muricholic, and ursodeoxycholic) in samples of Basal Bile and DAPM-Bile from four randomly chosen rats were assayed by high performance liquid chromatography (Hagey et al., 1998).

**Cytotoxicity.** An XTT (2,3-bis [2-carboxyethyl]-4,4-disulfonic acid)-tetrazolium-5-carboxanilide; tetrazolium salt)-based colorimetric assay (Roche Diagnostics, Indianapolis, IN) that measures the formation of a soluble formazan dye was used to assess metabolic activity of cells. Dye absorbance was read at 450 nm on a Biorad Microplate Reader Model 3500 UV (Hercules, CA). Data are expressed as optical density of formazan produced per μg DNA.

**DNA quantitation assay.** DNA was quantified using Hoechst 33258 (Molecular Probes, Eugene, OR) and the procedure of Rago et al. (1990). Each DNA measurement was based on a minimum of eight wells, with alternate columns of wells in plates used for endpoint assays versus DNA measurements.

\(^{14}\)C-DAPM uptake. \(^{14}\)C-DAPM-Bile and iso-NaCl were added to the apical and basolateral domains of monolayers, respectively. After 30 min, monolayers were rinsed, solubilized in 0.1 N NaOH and 1% SDS for 2 h at room temperature, mixed with scintillation cocktail (Betafluor, National Diagnostics, Manville, NJ), and counted in a Packard Tri-Carb 1900CA Liquid Scintillation Analyzer (Downers Grove, IL). Values are expressed as mmol total \(^{14}\)C/(DAPM + metabolite) uptake per μg DNA.

**TER measurements.** Confluent monolayers were placed in an Endohm-12 culture cup/chamber. TER measurements were taken at 1–5 min intervals using an EVOM Epithelial Voltohmeter (World Precision Instruments, Sarasota, FL). Changes in TER are expressed as a percentage of the baseline resistance.

**AMG uptake/AMG permeability.** AMG, a nonmetabolizable monosaccharide primarily transported by the Na\(^+\)-glucose cotransporter (Amsler and Cook, 1982), was previously used to evaluate the Na\(^+\)-dependent uptake of...
glucose by BEC (Lazaridis et al., 1997). Following exposure, monolayers on inserts were rinsed with glucose-free iso-NaCl, transferred to new wells, and 0.5 mM AMG (containing 0.5 mCi 14C-AMG) in glucose-free iso-NaCl was added to the apical chamber. Mannose (0.5 mM) was added to the basolateral chamber to maintain osmotic equilibrium. To assess AMG permeability, aliquots of basolateral solution were removed at various time points, mixed with scintillation fluid, and counted. To assess AMG uptake at various times, apical and basolateral solutions were rapidly replaced with ice-cold stop solution (0.5 mM phloridzin in iso-NaCl) for 15 min. Cells were then rinsed, solubilized for 2 h at room temperature, added to scintillation fluid, and counted. Values are expressed as nmol total AMG uptake or leakage per mg DNA. Negative controls consisted of incubating BEC monolayers with AMG at 4°C, and in the absence of sodium, by substituting tetramethylammonium chloride (TMA-Cl, [(CH3)4N]+Cl–) for sodium chloride.

**Cellular ATP.** ATP was measured using the ATP Bioluminescence Assay Kit HS II (Roche Diagnostics, Indianapolis, IN). After exposure, cells were lysed using a 1:1 solution of dilution and lysis buffers for 15 min at room temperature. Samples were further diluted 1:100 in dilution buffer prior to measurements on a Monolight 2010 luminometer (Analytical Luminescence Laboratory, San Diego, CA). Negative controls consisted of iso-NaCl, and positive controls consisted of 0.5 mM hydrogen peroxide (Andreoli and Mallett, 1997). Values are expressed as nmol ATP per μg DNA.

**MMP measurements.** MMP was measured using JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetracyanobenzimidazolylcarbocyanine iodide, Molecular Probes, Eugene, OR), a cationic dye that exhibits potential-dependent accumulation in mitochondria, by the method of Nuydens et al. (1993). Following exposure, cells were loaded with 10 μM JC-1 in iso-NaCl for 20 min at 37°C. Green fluorescence was measured first at excitation and emission filters of 485 and 530 nm, respectively, and then red fluorescence was measured at excitation and emission filters of 535 and 590 nm, respectively, on a Perkin Elmer HTS7000 Plus BioAssay Reader (Norwalk, CT) that uses HTSoft Version 2.0 software (Perkin Elmer, 1999) for integration. A reagent blank was subtracted from all samples. The ratio of measured fluorescence intensities at both wavelengths is an indication of the mitochondrial membrane potential (Cossarizza et al., 1993). Negative controls were exposure to iso-NaCl, and positive controls were exposure to 100 and 200 μM menadione (2-methyl-1,4-naphthoquinone), a known mitochondrial depolarization agent (Palmeira et al., 1996). Values are expressed as the ratio of green to red fluorescence per μg DNA.

**Electron microscopy.** BEC monolayers were fixed in 1% glutaraldehyde and 1% paraformaldehyde in 0.1 M cacodylate buffer (pH 7.2) for 1 h at room temperature, postfixed in 1% OsO4 in 0.1 M cacodylate buffer, rinsed, stained en bloc with 2% aqueous uranyl acetate (Andreoli and Mallett, 1997). Values are expressed as nmol ATP per μg DNA.

**Statistics.** Data are presented as mean ±SE and were analyzed by one-way ANOVA followed by Tukey’s post hoc test using SPSS 10.0 for Windows (San Diego, CA). If a significant difference between groups was indicated by ANOVA, the means for data at individual time points were compared between iso-NaCl and Basal-Bile treatments and between Basal-Bile and DAPM-Bile (1st Hr or 2nd Hr) treatments. Significance was accepted at a p value of ≤0.05.
RESULTS

Cytotoxicity of DAPM-Bile and DAPM Uptake Studies

Exposure of BEC monolayers to DAPM-Bile (1st Hr) or (2nd Hr) for 120 min caused cytotoxicity as indicated by ~75% and 50% less formazan formation, respectively (Fig. 2). In contrast, exposure to Basal Bile for 120 min had no cytotoxic effect (Fig. 2). To determine if the greater cytotoxic effect of DAPM-Bile (1st Hr) was correlated with a higher concentration of DAPM and its metabolites in bile, we measured biliary $^{14}$C concentrations and uptake of radioactivity by BEC, using pooled bile samples collected during the 1st Hr or 2nd Hr after treatment with $^{14}$C-DAPM. As shown in Table 1, DAPM-Bile (1st Hr) had ~55% higher concentrations of $^{14}$C-DAPM and its metabolites than DAPM-Bile (2nd Hr), and $^{14}$C uptake by BEC was ~110% greater from DAPM-Bile (1st Hr) than (2nd Hr).

The greater cytotoxicity of DAPM-Bile (1st Hr) could possibly be due to DAPM-induced secretion of a more cytotoxic bile acid profile by the liver. A precedent for this alternative is the report (Benedetti et al., 1997) that unconjugated, hydrophobic bile acids cause mitochondrial swelling and rarefaction in rat bile duct fragments in vitro without altering TJs or microvilli, while hydrophilic or conjugated bile acids had no effect. However, high performance liquid chromatography analysis showed no significant increases in hydrophobic bile acids or other changes in bile acid composition between Basal Bile and DAPM-Bile (1st Hr or 2nd Hr) (data not shown).

Effects of DAPM-Bile on TJ Integrity and Paracellular Permeability

Baseline TERs of BEC monolayers showed considerable variation (mean ± SE = 885 ± 111 ohms/cm$^2$). Thus, the influence of exposure was assessed as the percent change of a monolayer’s TER over time from its baseline value. During the first 30 min, BEC monolayers showed typical TER decreases of 2–5%, with gradual returns to steady state levels by 45–60 min, when exposed to either Basal Bile, DAPM-Bile (1st Hr) or (2nd Hr) (Table 2). TER values remained stable thereafter in monolayers exposed to Basal Bile. In contrast, TER values began to decline by 90 min after exposure to DAPM-Bile (1st Hr), with significant decreases of ~16% by 120 min (Table 2). DAPM-Bile (2nd Hr) produced variable effects on TER values with the percent change in baseline levels at 120 min ranging from 0 to 9% (Table 2).

Paracellular permeability of monolayers was unchanged by exposure to iso-NaCl and Basal Bile, as indicated by leakage of 2–3 nmol of AMG/μg DNA from the apical to the basolateral chamber and an EVOM Epithelial Voltohmeter®. Data represent the mean ± SE of a minimum of three experiments.

### TABLE 1

<table>
<thead>
<tr>
<th>Parameter measured</th>
<th>DAPM-Bile (1st Hr)</th>
<th>DAPM-Bile (2nd Hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DAPM/metabolite concentration in bile (mM)</td>
<td>8.6</td>
<td>5.6</td>
</tr>
<tr>
<td>Total DAPM/metabolite uptake into BEC (μmoles)</td>
<td>176 ± 6</td>
<td>79 ± 3</td>
</tr>
<tr>
<td>% DAPM/metabolite uptake by BEC</td>
<td>1.9 ± 0.08</td>
<td>0.9 ± 0.04</td>
</tr>
<tr>
<td>Normalized DAPM/metabolite uptake (mmol/μg DNA)</td>
<td>0.65 ± 0.03</td>
<td>0.31 ± 0.01</td>
</tr>
</tbody>
</table>

Note. DAPM-Bile samples were pooled from two rats administered 50 mg DAPM/kg containing ~8.5 μCi $^{14}$C-DAPM/rat (see Materials and Methods). Concentrations of DAPM and its metabolites in bile, and amounts absorbed by BEC were estimated from levels of $^{14}$C radioactivity measured by scintillation counting and calculated using the molecular weight of DAPM. Data represent the mean ± SE of 3 experiments.

### TABLE 2

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Basal-Bile</th>
<th>DAPM-Bile (1st Hr)</th>
<th>DAPM-Bile (2nd Hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>30</td>
<td>4.1 ± 3.2</td>
<td>2.4 ± 1.0</td>
<td>4.4 ± 2.2</td>
</tr>
<tr>
<td>60</td>
<td>0.7 ± 1.2</td>
<td>2.6 ± 3.2</td>
<td>1.9 ± 1.2</td>
</tr>
<tr>
<td>90</td>
<td>0.4 ± 0.9</td>
<td>7.2 ± 3.0</td>
<td>1.6 ± 1.4</td>
</tr>
<tr>
<td>120</td>
<td>1.0 ± 1.4</td>
<td>15.6 ± 4.6*</td>
<td>3.8 ± 4.1</td>
</tr>
</tbody>
</table>

Note. TERs of BEC monolayers were determined using an Endohm-12 culture chamber and an EVOM Epithelial Voltohmeter®. Data represent the mean ± SE of a minimum of three experiments. *$p < 0.05$ compared to basal bile.
Effects of DAPM-Bile on Na\(^+\)-Dependent Glucose Transport

Uptake of AMG was decreased by ~40% and 25%, respectively, when BEC monolayers were exposed to DAPM-Bile (1st Hr) or (2nd Hr) for 60 min (Fig. 3A). No further changes in AMG uptake were observed with a longer exposure of 120 min (Fig. 3A). To verify that AMG transport was energy- and Na\(^+\)-dependent, BEC monolayers were incubated with AMG at 4\(^\circ\)C, and in the absence of Na\(^+\) (TMA-Cl substituted for NaCl). As expected, uptake under these conditions was impaired by ~95% and 92%, respectively (Fig. 4).

Glucose levels (mean ± SE) in pooled DAPM-Bile (1st Hr) and (2nd Hr) samples used in these experiments were 11 ± 3 and 23 ± 6* mg/dl, which is ~4- and 8-fold greater, respectively, than the 3 ± 1 mg/dl levels in Basal Bile (*p < 0.05 compared to Basal Bile). These increases are consistent with prior observations of a rapid elevation in biliary glucose following DAPM administration (Kanz et al., 1992; 1998). To rule out variations in biliary glucose as a factor for the observed differences in AMG uptake shown in Figure 3A, Basal Bile samples were supplemented with D-glucose to final concentrations of ~10 and ~17 mg/dl, which was equal to or greater than the glucose levels (~11 mg/dl) found in DAPM-Bile (1st Hr). The presence of high glucose concentrations in Basal Bile did not alter subsequent AMG uptake by BEC (Fig. 4).

Effects of DAPM-Bile on ATP Content and Mitochondrial Membrane Potential

Initial studies investigated potential effects of vehicle treatment (2 ml/kg of 35% ethanol per os) on BEC cellular ATP levels following vehicle-bile exposure because Devi et al. (1993) reported that exposure of fetal hepatocytes in vitro to 2 mg/ml (0.2%) ethanol caused structural and functional changes to mitochondria that included decreases in cellular ATP levels. Figure 5A shows that exposure of BEC to bile from vehicle-treated rats did not alter ATP levels through 120 min compared to BEC exposed to Basal Bile or iso-NaCl. Surprisingly though, exposure of BEC to Basal Bile from rats before treatment with DAPM (Fig. 5B) produced...
30% decreases in ATP levels at 15 and 30 min. This decrease, however, did not appear to be due to an injurious process, because ATP levels returned to normal by 60 min. We are presently unable to explain this discrepancy between Basal Bile samples from different groups of rats, because procedures for surgery, equilibration, and basal bile collection were equivalent for all rats irrespective of subsequent treatment with vehicle or DAPM. One possible explanation could be that exposure of BEC in vitro to bile produces minor alterations such as decreases in TERs (Table 2) and ATP levels (Fig. 5B) that return to normal within 15–30 min, and that cellular equilibration occurred more rapidly in the experiments of Figure 5A than 5B.

Exposure of BEC to DAPM-Bile (1st Hr) and (2nd Hr) caused ATP depletions of ~55% and 30% by 30 min, which was further depleted to ~85% and 55%, respectively, by 60 min (Fig. 5B). Mean ATP values (nmol/µg DNA) for monolayers at 60 min were Basal Bile, 149.4 ± 8.8; DAPM-Bile (1st Hr), 18.8 ± 1.1; and DAPM-Bile (2nd Hr), 69.5 ± 4.0 (Fig. 5B). As expected, the positive control, hydrogen peroxide, caused a rapid and nearly complete depletion of ATP in BEC by 15 min (0.9 ± 0.03 nmol/µg DNA), which continued to decline through 60 min (0.4 ± 0.01 nmol/µg DNA).

Figure 6 shows that exposure of BEC to DAPM-Bile (1st Hr) and (2nd Hr) for 15 min induced an ~2-fold increase in the green to red fluorescence ratio of JC-1 (24.5 ± 3.5 and 22.0 ± 1.9, respectively) compared to Basal Bile (11.0 ± 0.9). By 60 min, mitochondrial depolarization induced by DAPM-Bile (1st Hr) or (2nd Hr) was equivalent to that observed with the higher concentration of the positive control, menadione.

**Effects of DAPM-Bile on BEC Ultrastructure**

Ultrastructural examination of BEC monolayers exposed to DAPM-Bile (1st Hr) revealed striking similarities to the in vivo mitochondrial injury previously described (Kanz et al., 1998). BEC exposed to Basal Bile for 120 min displayed conventional ultrastructure: mitochondria with dense matrices, lobate nuclei, and intact microvilli on the apical surface (Fig. 7A). In contrast, BEC exposed to DAPM-Bile (1st Hr) for 30 min displayed swollen mitochondria with translucent matrices and fewer microvilli on the apical surface (Fig. 7B). By 120 min, BEC exposed to DAPM-Bile (1st Hr) displayed severely damaged mitochondria, a nearly complete absence of apical microvilli, and loss of nuclear heterochromatin (Fig. 7C).
DISCUSSION

Biliary epithelial cells comprise 3–5% of the liver (Ludwig et al., 1998); thus, identifying the mechanisms by which drugs and chemicals damage BEC has been difficult. Development of culture methods that produce polarized BEC populations from rodents and humans with morphological and functional characteristics analogous to those reported for BEC in vivo (Ishida et al., 2001; Yang et al., 1993) has provided new approaches to the study of biliary injury. Our model shows promise for investigating impairments in cholangiocyte biology caused by drugs/metabolites excreted in bile, because polarized BEC in vitro demonstrated phenotypic characteristics similar to BEC in vivo, as exemplified by the lack of injury in BEC monolayers exposed to normal rat bile (Figs. 1 and 7) and by the ability of “toxicant-bile” (bile that injures BEC in vivo) to produce a pattern of morphological damage and cytotoxicity (Figs. 1 and 7) consonant with that observed in vivo (Kanz et al., 1995; 1998). Importantly, BEC monolayers are sufficiently stable to dissect the time course of injurious events caused by toxicants on various BEC functions, such as glucose transport, TJ permeability, and mitochondrial activity (Figs. 2, 3, 5, and 6).

We used bile collected from DAPM-treated rats in our model because prior studies had demonstrated that the proximate toxicant(s) of DAPM was excreted in bile (Kanz et al., 1995) and that the concentrations of DAPM/metabolites varied appreciably with time (Dugas et al., 2001). The use of bile as a toxicant delivery method was validated by our observations of a dose-dependent effect (Table 1) of 1st Hr versus 2nd Hr DAPM-Bile on cytotoxicity (Fig. 1) and ATP depletion (Fig. 5).

We provide solid evidence for a temporal sequence of events in DAPM-induced injury of BEC. The time-course studies demonstrated that mitochondrial MMP ratios increased and ATP levels decreased as early as 30 min after exposure to DAPM-Bile (Figs. 5B and 6), while impairment of glucose uptake via a Na\(^+\)-dependent pathway required 60 min of exposure (Fig. 3A), and impairment of TJ permeability required 90 to 120 min of exposure to DAPM-Bile (Figs. 2 and 3B). Ultrastructural observations (Fig. 7) also provided convincing support that injury to mitochondria occurred prior to morphological alterations in BEC plasma membranes (e.g., loss of microvilli). Thus, BEC mitochondrial injury preceded impairments in Na\(^+\)-dependent glucose transport and TJ permeability. Therefore, early mitochondrial dysfunction with subsequent ATP depletion may play a major role in the later impairments in glucose uptake and TJ permeability. However, confirmation of this premise requires detailed investigation.

The effects of ATP depletion on BEC function and membrane structure have been examined by Doctor et al. (1999; 2000), using 120 min of ischemia in vivo or severe metabolic depletion of ATP in vitro. A striking structural effect was a loss of BEC plasma membrane surface area with diminished basolateral interdigitations between BEC, fewer apical microvilli, and a dissociation of the actin-membrane linking protein, ezrin.
from the microvillar cytoskeleton. Depletion of ATP by >95% caused TERs to diminish by ~70%, but TJ integrity was considered maintained because ruthenium red permeation was not observed within tight junctions by electron microscopy (Doctor et al., 1999). In our studies, BEC monolayers treated with DAPM-Bile exhibited 60–90% deficits in ATP levels (Fig. 5B), decreased numbers of apical microvilli (Fig. 7), and increased TJ permeability (AMG leakage from apical to basolateral media, Fig. 3), with only minor decreases in TERs (Fig. 2). The more substantial paracellular permeability observed by us compared to the studies by Doctor et al. (1999) could have been due to our use of AMG as a marker, because it is ~4-fold smaller than ruthenium red, and is assessed via scintillation counting rather than observed by electron microscopy.

BEC lower biliary glucose levels by both Na\(^+\)-dependent and Na\(^+\)-independent pathways (Lira et al., 1992). A Na\(^+\)-dependent SGLT1 transporter has been described on the apical domain of BEC, which resorbs glucose via energy dependent transport of Na\(^+\) up its electrochemical gradient by Na\(^+\), K\(^+\)-ATPase (Lazaridis et al., 1997). In our model of polarized BEC, glucose [AMG] uptake was found to be both temperature sensitive and Na\(^+\) dependent (Fig. 4), which is consistent with a role for SGLT1. Membrane proteins reported by Doctor et al. (2000) to be internalized in ATP-depleted BEC were SGLT1 and Na\(^+\), K\(^+\)-ATPase. Thus, the diminished glucose [AMG] uptake that we observed in BEC exposed to DAPM-Bile in vitro and the early rise in biliary glucose found after in vivo DAPM treatment could be due to the absence of SGLT1 and Na\(^+\), K\(^+\)-ATPase on BEC apical membranes secondary to ATP depletion.

But an alternative explanation for the observed decrease in AMG uptake could be a direct effect of DAPM/metabolites on membrane proteins, since other compounds such as platinum disrupt glucose transport by direct binding to the transporter (Potdevin et al., 1998), while highly lipophilic compounds such as pentachlorophenol and 1,1,2,2-tetrachloroethane inhibit glucose transport and Na\(^+\), K\(^+\)-ATPase by disruption of lipid bilayer properties (Cascorbi and Foret, 1991). These studies did not address the elevation in biliary P\(_i\), which occurs concurrently with the glucose rise after DAPM administration in vivo. However, if BEC have P\(_i\) transporters analogous to those found on the apical membranes of cells of the kidney and small intestines (Murer et al., 2001), either indirect effects such as ATP depletion or direct effects of DAPM/metabolites on BEC P\(_i\) transporters would explain the concurrent rise in biliary P\(_i\) and glucose. These possibilities will require more in-depth studies of glucose and phosphate kinetics in BEC exposed to DAPM-Bile.

Prior in vitro studies of the effects of chemicals/drugs on BEC are rare, and most used freshly isolated cells to assess cytotoxicity via leakage of enzymes or trypan blue exclusion. Parola et al. (1990) reported that BEC showed dose- and time-dependent cell death following exposure to menadione and cumene hydroperoxide but not to carbon tetrachloride and 7-ethoxy coumarin. Both oxidant compounds rapidly depleted glutathione levels in BEC followed by oxidation of protein thiols. The absence of an injurious effect by carbon tetrachloride and 7-ethoxy coumarin to BEC was attributed to the inability of BEC to bioactivate these compounds to toxic intermediates (Parola et al., 1990). In studies mimicking reperfusion injury (anoxia/reoxygenation), isolated BEC were more resistant to cell death by anoxia but less resistant to cell death by reoxygenation than hepatocytes. During reoxygenation, BEC were found to produce reactive oxygen species at a rate ~4 times greater than hepatocytes (Noack et al., 1993). Hill et al. (1999) showed that BEC cytotoxicity in vitro occurred at concentrations of ANIT comparable to that found in bile, and that conditioned media from BEC exposed to ANIT caused neutrophil-dependent hepatocyte injury in hepatocyte/neutrophil co-cultures. These studies demonstrate the utility of in vitro models for examining mechanisms of BEC cytotoxicity, but freshly isolated cells lack validity with regard to barrier integrity, absorption functions, and other normal phenotypic characteristics of BEC in vivo.

To our knowledge, only one prior study has examined the cytotoxic effects of chemicals/drugs on BEC monolayers. Lakehal et al. (2001), using gallbladder-derived BEC grown on collagen from 19 patients, showed that the drug flucloxacillin was not injurious to BEC, whereas conditioned medium from hepatocytes exposed to flucloxacillin or supernatant from microsomes incubated with flucloxacillin caused dose-dependent loss of viability in ~50% of the human BEC populations. Lakehal et al. (2001) concluded that their findings supported the concept that exposure of BEC to biliary metabolites produced by hepatocytes may be responsible for drug-induced cholangiopathies. Consequently, our model—in which polarized BEC maintain apical and basolateral surfaces, bile exposure occurs only via the apical surface, and bile is used as a vehicle for delivery of drug/chemical metabolites physiologically metabolized and excreted from rat hepatocytes—is a major step up from previous methods for assessing toxicity in the biliary tree and will be especially advantageous in analyzing potential, new drugs for injurious effects to BEC.

In summary, we have developed an in vitro BEC/bile exposure model that closely mimics the morphological and functional injury produced by DAPM in vivo. This model allowed us to dissect the temporal sequence of DAPM-induced alterations in BEC: DAPM-Bile first alters BEC mitochondrial structure and function, then BEC glucose transport is impaired, and later, alterations in tight junctions become apparent. Now that the proof of concept has been established, this model can be used to address the mechanistic basis for injury by DAPM and other BEC toxicants.

A recent report by Frei et al. (Am. J. Physiol. Gastrointestinal. Liver Physiol., 24 Nov. 2004-Epub ahead of print) provides support for inorganic phosphate uptake from bile by transporters on hepatocyte and cholangiocyte apical membranes. These authors report sodium-dependent P\(_i\) uptake by canaliculal liver plasma membrane vesicles, localization of P\(_i\) transporters...
at hepatocyte canalicular membranes and cholangiocyte brush border membranes by immunofluorescence, and identification of the putative apical transporter as NaPi-IIb by RT-PCR and Western blotting.

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