Oxidative Stress Induces Internalization of the Bile Salt Export Pump, Bsep, and Bile Salt Secretory Failure in Isolated Rat Hepatocyte Couplets: A Role for Protein Kinase C and Prevention by Protein Kinase A

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We have shown that Ca\(^{2+}\)-mediated protein kinase C (PKC) activation induces impairment of bile salt secretory function and F-actin redistribution in hepatocyte couplets. Because oxidative stress induces Ca\(^{2+}\) elevation, we tested here whether PKC inhibition or protein kinase A (PKA) activation, which often counteracts PKC-dependent effects, can prevent and reverse these alterations. The pro-oxidant compounds tert-butylhydroperoxide (tBOOH, 100 μM) and 2,3-dimethoxy-1,4-naphthoquinone (30 μM), reduced by −41% and −29%, respectively, the percentage of couplets accumulating the fluorescent bile salt analog, cholylysylfluorescein in their canalicular vacuoles (p < 0.01). tBOOH-induced bile salt secretory failure was accompanied by internalization of the canalicular bile salt export pump (Bsep), and disarrangement of cytoskeletal F-actin. All these deleterious effects were fully prevented by the intracellular Ca\(^{2+}\) chelator BAPTA/AM (20 μM), the pan-specific PKC inhibitors H7 (100 μM) and staurosporine (1 μM), the inhibitor of Ca\(^{2+}\)-dependent PKCs, G66976 (2 μM), and the PKA activator dibutyryl-cAMP (500 μM). H7, G66976, and dibutyryl-cAMP not only prevented but also fully reversed the decrease in the cholylysyl-fluorescein accumulation. In conclusion, these results suggest that low levels of oxidative stress impair bile salt secretion by internalizing Bsep through a Ca\(^{2+}\)-dependent, PKC-mediated mechanism, and that inhibition of PKC, or activation of PKA, prevents and reverses these effects. Alterations in actin organization may be a causal factor.

Key Words: systems toxicology, oxidative injury; biotransformation and toxicokinetics; biliary excretion; xenobiotic transporters; in vitro alternatives; hepatocytes; systems toxicology; signal transduction.

INTRODUCTION

Oxidative stress induces cellular injury by excessive production of reactive oxygen species (ROS) of high reactivity against DNA, lipids, and proteins (Cesaratto et al., 2004; Poli et al., 2004). A role for oxidative stress either as a primary causal agent or as an aggravating factor in hepatopathies has become increasing apparent, including ischemic liver, alcoholism, non-alcoholic steatosis, and its progression to steatohepatitis, cholestasis, and pathologies leading to hepatic accumulation of heavy metals, such as hemochromatosis (iron) and Wilson’s disease (copper) (Cesaratto et al., 2004).

In previous studies, we described a number of early hepatobiliary dysfunctions induced by low, non-necrotic levels of oxidative stress, which are relevant to bile formation. Using isolated rat hepatocyte couplets, an in vitro model for the study of polarized plasma-bile transport, we showed that low doses of the synthetic hydroperoxide, tert-butylhydroperoxide (tBOOH) (Ahmed-Choudhury et al., 1998), or the redox-cycling quinones, menadione and 2,3-dimethoxy-1,4-naphthoquinone (DMNQ) (Stone et al., 1996), induce a dose-dependent decrease in the hepatocyte couplet ability to secrete apically the fluorescent bile salt analog, cholylysylfluorescein (CLF), at concentration not affecting either oxidized glutathione or ATP intracellular levels (i.e., under low oxidative stress conditions). These alterations were accompanied by plasma membrane bleb formation and disarrangement of the F-actin cytoskeleton (Ahmed-Choudhury et al., 1998; Stone et al., 1996). However, the mechanism/s underlying these alterations remained unidentified.

Recent studies on crosstalk between signaling pathways and oxidative stress have demonstrated that reactive oxygen species...
(ROS) induce both short- and long-term changes in cellular function by affecting signal transduction mechanisms. This may occur by modulating phosphorylation status of cellular targets relevant to cell function and by influencing activity of transcription factors, respectively (Cesaratto et al., 2004; Poli et al., 2004). Among the multiple signaling pathways that are thought to mediate ROS-induced damage, the protein kinase C (PKC)-dependent pathway seems to play a key role. Protein kinase C contains unique structural features that make it susceptible to oxidative modification, or redox-dependent activation (Kamata and Hirata, 1999). The N-terminal regulatory domain contains zinc-binding, cysteine-rich motifs that are readily oxidized by ROS; when oxidized, the autoinhibitory function of the regulatory domain is compromised and, consequently, cellular PKC activity is stimulated. Moreover, under pro-oxidant conditions, tyrosine phosphorylation of PKC occurs, leading to enhanced enzymatic activity. Formation of fatty acid hydroperoxy derivatives, the first event in lipid peroxidation, stimulates PKC activity further. Finally, cytosolic Ca\(^{2+}\) elevations occur under oxidative stress conditions, and the so-called classical PKC isoforms are activated by low Ca\(^{2+}\) concentrations. Alternatively, oxidative stress may modulate PKC activity by stimulating protein kinases acting at an upstream level, such as phosphoinositide 3 kinase (Kang et al., 2002), which activates Ca\(^{2+}\)-dependent PKCs in hepatocytes (Carini et al., 2004). It is therefore not surprising that PKC is involved in a number of hepatocellular alterations occurring under oxidative stress conditions, such as apoptosis induced by bile salts (Jones et al., 1997), or lipid peroxidation and membrane damage induced by pro-oxidant compounds, including tert-butylhydroperoxide (tBOOH) (von Ruecker et al., 1989), H\(_2\)O\(_2\) (von Ruecker et al., 1989), and bile salts (Borgognone et al., 2005).

Protein kinase C activation mediates a number of subtle alterations at the level of hepatocellular structures involved in biliary secretion. We have shown that hormonal modulators known to activate PKC induce hepatocyte plasma membrane bleb formation associated with disarrangement of actin cytoskeleton; this was accompanied by impairment in the couplent capability to secrete acically the fluorescent bile salt, CLF (Roma et al., 1998). These results are in line with more recent studies showing that Ca\(^{2+}\)-dependent PKC activation impairs bile salt excretion in isolated rat perfused liver, in association with retrieval of the bile salt export pump (Bsep, Abcb11) (Kubitz et al., 2003b). This carrier belongs to the superfamily of ATP-binding-cassette transporters, and it is involved in the ATP-dependent, rate-limiting canalicular transport of C24-amidated BSs conjugated with glycine or taurine (Arrese and Ananthanarayanan, 2004).

Because PKC can be activated under oxidative stress conditions, and because PKC activation is associated with bile salt secretory failure and Bsep retrieval, we assessed here whether the two events are causally related. For this purpose, we used tBOOH and DMNQ as two different oxidative stress inducers, because their pro-oxidant hepatic metabolisms occur by dissimilar pathways; tBOOH is mainly converted into peroxyl and alkoxyl free radicals by microsomal cytochrome P450 (Davies, 1989), whereas redox cycling quinones like DMNQ are mainly reduced by mitochondrial NADH:ubiquinone oxidoreductase to a semiquinone radical, which is reoxidized back to quinone by oxygen, thus continuously generating ROS (Powis et al., 1981).

Our results show that under low oxidative stress levels, failure of bile salt excretion occurs, in association with retrieval of Bsep from the canalicular membrane into vesicular structures. These alterations were fully accounted for by activation of Ca\(^{2+}\)-dependent PKC isoforms and counteracted by protein kinase A (PKA) activation. Finally, these phenomena correlated closely with integrity of the actin cytoskeleton, a factor known to support the normal localization of canalicular transporters (Rost et al., 1999).

### MATERIALS AND METHODS

**Materials.** CLF was kindly provided by Dr. Charles O. Mills (Birmingham, UK). Collagenase type A from *Clostridium histolyticum* was purchased from Gibco (Paisley, UK). Leibovitz-15 tissue culture medium, BSA (fraction V), tBOOH, N\(_6\),2′-o-dibutyryladenosine 3′,5′-cyclic monophosphate (DB-cAMP), 1-(5-isouquinolinylsulfonyl)-2-methyl-piperazine (H\(_7\)), staurosporine (SP), DMNQ, DMSO, and FITC-labeled phalloidin were obtained from Sigma Chemical Co. (Poole, Dorset, UK). KT5720, BAPTA/AM and Go6976 were obtained from Regis (Birmingham, UK) and Calbiochem (San Diego, CA). All other chemicals were of reagent grade.

**Animals.** Male Wistar rats (210–250 g) were used throughout. Animals were maintained on a standard laboratory diet (41B maintenance diet, Pilsbury, Birmingham, UK) and tap water ad libitum. Rats were anesthetized with Ketamine hydrochloride (Ketalar®; 6 mg/100g body wt, i.p.), with Medetomidine (Domitor®; 25 μg/100g body wt, i.p.). All animals received humane care according to the criteria outlined in the Guide for the Care and Use of Laboratory Animals prepared by the National Academy of Sciences and published by the National Institutes of Health (NIH publication 86–23, revised 1985).

**Coupel isolation, enrichment, and culture.** Hepatocyte couplets were obtained according to the 2-step collagenase perfusion procedure followed by centrifugal elutriation for further enrichment from singles and multiplets, as described by Wilton et al. (1991). The resulting preparation, containing 71 ± 3% of couplets of high viability (>97%), was plated in Leibovitz-15 medium containing 50 U/ml penicillin and 50 μg/ml streptomycin onto 35-mm plastic culture dishes (2 ml/dish) at a density of 5 × 10\(^4\) units/ml, and incubated at 37°C for 4.5 h.

**Evaluation of tBOOH-induced lipid peroxidation.** The magnitude of the oxidative stress levels induced by tBOOH was evaluated by measuring generation of lipid peroxidation products. Lipid peroxidation was evaluated by a modification of the thiobarbituric acid-reactive substances (TBARS) method (Buege and Aust, 1978), with minor modifications (Borgognone et al., 2005). A standard curve using 1,1,3,3-tetramethoxypropane, which is converted to a semiquinone radical by oxygen, thus continuously generating ROS (Powis et al., 1981).

**Assessment of bile salt secretion.** Bile salt secretory function was evaluated by assessing the proportion of couplets (>50) performing canalicular
vacuolar accumulation (cVA) of the fluorescent bile salt analog CLF (Ahmed-Choudhury et al., 1998; Roma et al., 1998); this compound is similar in both uptake kinetics parameters and secretory properties to its naturally occurring bile salt parent compound, cholyglycine, and it is excreted into the bile canaliculus via Bsep (Mills et al., 1999).

To carry out cVA of CLF, the fluorescent bile salt analog (2 μM) was added to each plate and incubated at 37°C for 15 min. The CLF was then removed from the media by washing twice with Leibovitz-15 medium, and cVA of CLF was immediately assessed by using an inverted fluorescent microscope (Olympus IMT2-RFL, Olympus Optical Ltd., London, UK).

Assessment of Bsep and F-actin localization. For Bsep immunostaining, couplets on coverslips were fixed with 3% paraformaldehyde for 15 min at room temperature, washed three times with PBS, quenched with 50 mM NH4Cl in PBS, and washed again. Next, the cells were treated with a permeabilizing buffer (0.2% Triton X-100 in PBS containing 0.3% wt/vol BSA) for 10 min, before a new PBS wash and further incubation with 1.5% goat serum and 1.5% BSA in PBS for 30 min, at room temperature. Cells were then incubated for 40 min with anti-Bsep rabbit antibody 1:250 (kindly provided by Dr. Michael Müller), followed by a further incubation with FITC-labeled goat anti-rabbit IgG, 1:100 for 40 min.

Phalloidin-FITC labeling of fixed, permeabilized cells was employed to visualize cellular distribution of the F-actin cytoskeleton. Fluorescent staining of actin was performed as described elsewhere (Roma et al., 1998). Briefly, cells were fixed with 3% formalin in PBS and stored at 4°C until permeabilized with 0.1% Triton X-100 in PBS. F-actin was labeled by treating the cells with phalloidin-FITC (5 μg/ml in PBS). After the cells were washed in PBS, they were mounted in 90% glycerol-PBS containing 2.5% (wt/vol) diazabicyclo[2.2.2]octane.

Once stained, the specimens for both Bsep and F-actin analysis were examined under fluorescent light (excitation wavelength 490 nm, emission wavelength >525 nm) in a Zeiss microscope (Axiovert 350TV; Carl Zeiss Oberkochen Ltd., Welwyn Garden City, Herts, U.K.), equipped with Zeiss plan-neofluar lenses. Monochrome images taken in 1-μm steps were captured on a CCD video camera (Hamamatsu Photonic Sys. Corp., Hamamatsu City, Japan), and out-of-focus-flair was removed using a deconvolution program (Micro-Tome Mac, Vaytek Ltd., Fairfield, IL).

Image analysis. Images were analyzed to quantify the total intensity of fluorescence within the couplet and, specifically, in the canaliculal region (for Bsep) or in the pericanalicular region (for F-actin). Total and (peri)canalicular fluorescence were quantified by using Openlab Digital Imaging software (Improvision, Warwick Science Park, Coventry, U.K.), as indicated elsewhere (Roma et al., 1998).

Treatments and experimental design. In prevention studies aimed to assess the involvement of transductional cascades in oxidative stress–induced hepatocanalicular dysfuncion, a number of signaling modulators were pre-administered for 15 min. They included the pan-specific PKC inhibitors H7 (100 μM) and SP (1 μM), the inhibitor of Ca2+-dependent PKCs Gö6976 (2 μM), and the PKA activator DB-cAMP (500 μM), with or without a 15-min pretreatment with the PKA inhibitor KT5720 (5 μM). These compounds were dissolved in DMSO, except for SP, which was dissolved in saline, and added in volumes ranging from 2 to 10 μl. After pre-treatment with these compounds, either tBOOH (100 μM) or DMNQ (30 μM) was added and exposed to the cells for 15 min. After these treatments, cVA of CLF or Bsep/F-actin localization was assessed, as indicated above.

In reversal studies aimed to assess the capability of different signaling modulators to reverse the deleterious effect of tBOOH on bile salt secretory function, cells were first exposed to tBOOH (100 μM) for 15 min, and CLF (2 μM) was then allowed to accumulate for a further 15-min period. Next, tBOOH was removed by replacement of the medium with tBOOH-free medium containing CLF and either H7 (100 μM), Gö6976 (2 μM), or DB-cAMP (500 μM). The cVA of CLF was then assessed every 10 min.

In ancillary experiments, viability before and after each treatment was assessed by the trypan blue exclusion test. After 15 min of administration, neither tBOOH nor DMNQ had any effect on hepatocellular viability, whether administered alone or in combination with the signaling modulators used in this study. Similarly, LDH release into the incubation medium was not affected by either of the compounds tested (data not shown).

Statistical analysis. Data were expressed as mean ± SE. Multiple means were compared with one-way analysis of variance (ANOVA), followed by Newman-Keuls multiple-range test for pairwise comparisons, using a computer program (PHARM/PCS, MicroComputer Specialist, Philadelphia). Differences were considered significant when p values were < 0.05.

RESULTS

Prevention of tBOOH-Induced Impairment of Bile Salt Secretory Function by PKC Inhibition

Under control conditions, the percentage of couplets exhibiting cVA of CLF was 71.8 ± 2.4%. As shown in Figure 1, cVA of CLF was not significantly affected by either of the pan-specific PKC inhibitors used, H7 or SP; when administered alone, tBOOH reduced both parameters significantly, and this inhibition was fully prevented by both PKC inhibitors. The tBOOH deleterious effect can be attributed to its ability to induce oxidative stress after metabolic conversion, as an increase of 200% in TBARS formation was recorded 15 min after its administration (from 0.6 ± 0.2 to 1.8 ± 0.5 nmol of MDA/mg of protein, p < 0.025).

Because oxidative stress leads to elevation of cytosolic Ca2+ (Orrenius et al., 1992), and synergistic action of Ca2+-is required for activation of the so called classical PKCs (Nishizuka, 1986), we analyzed whether Ca2+-mediated activation of these PKC isoenzymes is involved in the harmful effect induced by tBOOH. Both the intracellular Ca2+ chelator BAPTA/AM and the specific inhibitor of Ca2+-dependent
PKCs, G66976, fully prevented the decrease induced by tBOOH in cVA of CLF (Fig. 2). When administered alone, neither BAPTA/AM nor G66976 induced per se any effect on cVA of CLF.

Prevention of tBOOH-Induced Impairment of Canalicular Function and Bleb Formation by DB-cAMP Administration

PKC- and PKA-mediated signaling pathways often counterbalance each other to elicit regulated cellular responses (Yoshimasa et al., 1987). Therefore, we analyzed whether PKA activation induced by the permeant cAMP analog, DB-cAMP, prevents tBOOH-induced hepatocellular damage. As shown in Figure 3, DB-cAMP significantly counteracted tBOOH-induced reduction of cVA of CLF. This protective effect seems to be mediated by PKA activation, as suggested by the lack of protection when the PKA inhibitor, KT5720, was pre-administered.

Prevention of DMNQ-Induced Impairment of Canalicular Function and Bleb Formation by PKC Inhibition, Intracellular Ca²⁺ Sequestration, or DB-cAMP Administration

The redox-cycling quinone DMNQ was used as an alternative oxidative-stress inducer because its pro-oxidant metabolisms occur by a completely different metabolic pathway from that of tBOOH (Powis et al., 1981). At the dose employed here (30 µM), this redox-cycling quinone increased TBARS formation by 143% (from 0.7 ± 0.2 to 1.7 ± 0.3 nmol of MDA/mg of protein, p < 0.01).

As shown in Figure 4, DMNQ induced a reduction in cVA of CLF and, like that which occurred when tBOOH was used as a pro-oxidant, this alteration was prevented by the PKC inhibitor SP, the intracellular Ca²⁺ sequestering agent BAPTA/AM, and the PKA activator DB-cAMP.

Reversal of tBOOH-Induced Impairment of Canalicular Function by PKC Inhibition or DB-cAMP Administration

The time-course of the reversal by H7, G66976, and DB-cAMP of tBOOH-induced cVA of CLF impairment is shown in Figure 2.
Figure 5. A 15-min period of tBOOH exposure induced extensive impairment of cVA of CLF. Subsequent administration of H7, Gö6976, or DB-cAMP resulted in reversal of this effect. The recovery patterns for each were similar in time-scale, reaching virtually basal values from 40–50 min, onwards. Control experiments in which couplets pretreated with tBOOH were subsequently exposed to DMSO showed no spontaneous recovery of cVA of CLF.

 Prevention of tBOOH-Induced Redistribution of Bsep and F-Actin by PKC Inhibition or DB-cAMP Administration

Figure 6 shows phase contrast images and fluorescence images displaying Bsep distribution in typical, untreated couplets and in couplets exposed to tBOOH, either with or without H7 or DB-cAMP pretreatment. In control couplets, Bsep was mainly confined to the canalicular membrane. tBOOH induced plasma membrane blebbing (as visualized by phase contrast) and relocation of Bsep into vesicles localized all over the couplet body. Redistribution of Bsep was fully prevented by both H7 and DB-cAMP. None of these signal modulators had independent effects on Bsep localization (data not shown).

F-actin distribution under the same conditions used to study Bsep localization is shown in Figure 7. Cellular topology, as visualized by phase contrast microscopy, is also shown. In control couplets, F-actin displayed a pericanalicular location, forming a dense belt below the canalicular membrane. In tBOOH-treated couplets, extensive plasma membrane bleb formation occurred, and it was accompanied by redistribution of F-actin from the pericanalicular area to the cell body and the sinusoidal plasma membrane, particularly in the areas where blebs occur. As with Bsep, redistribution of F-actin was fully prevented by both H7 and DB-cAMP. None of these signal modulators had independent effects on either cellular topology or localization of F-actin (data not shown).

Quantitative evaluation of the proportion of Bsep fluorescence (in the canalicular membrane) or F-actin (in the pericanalicular area) is depicted in Figure 8. Results were in
line with those described qualitatively in the representative microphotographs depicted in Figures 6 and 7; i.e., 
\( t \text{BOOH} \) induced relocalization of Bsep and F-actin from the canalicular and the pericanalicular region, respectively, to the remaining cell body, and both H7 and DB-cAMP fully prevented these rearrangements.

**DISCUSSION**

In this study, a number of alterations in hepatocellular function and structure were induced by low oxidative stress levels in the couplet model, namely, (1) a reduced ability to accumulate the fluorescent bile salt analog CLF in the canalicular vacuole, and (2) disarray of the F-actin cytoskeleton. These results agree with previous findings in couplets (Ahmed-Choudhury *et al.*, 1998; Stone *et al.*, 1996) and in isolated perfused rat liver (Ballatori and Truong, 1989), where 
\( t \text{BOOH} \) was used as the oxidizing compound. However, the mechanisms underlying these alterations remained largely speculative from previous studies.

Impairment in bile salt secretory function was attributed to an oxidative stress-induced increase in oxidized glutathione, which inhibits bile salt transport in canalicular membrane vesicles (Griffiths *et al.*, 1987). However, this seems not to apply for 
\( t \text{BOOH} \) or DMNQ at the concentrations used here (100 and 30 \( \mu \text{M} \), respectively), because no change in cellular glutathione disulfide (GSSG) has been reported at these concentrations (Ahmed-Choudhury *et al.*, 1998; Stone *et al.*, 1996). Furthermore, at these concentrations, neither 
\( t \text{BOOH} \) (Ahmed-Choudhury *et al.*, 1998) nor DMNQ (Stone *et al.*, 1996) increases the release of the cytosolic enzyme lactate dehydrogenase or induces a reduction in ATP levels. Therefore more subtle cholestatic mechanisms must be operating under our mild oxidative stress conditions.

A change in Bsep activity due to internalization from the canalicular membrane is a possible causal factor. Bsep retrieval occurs in several cholestatic conditions, such as those induced by hyperosmotic shock, obstructive cholestasis, and administration of the cholestatic agents tauroliothoholate, estradiol-17\( \beta \)-D-glucuronide, and cyclosporin A (Crocenzi *et al.*, 2004). Furthermore, another member of the superfamily of ATP-binding-cassette transporters, the multidrug resistance-associated protein 2 (Mrp2), suffers retrieval following 
\( t \text{BOOH} \) exposure to isolated perfused rat livers (Schmitt *et al.*, 2000). Our results
on Bsep immunolocalization in tBOOH-treated couplets confirms that a similar phenomenon also applies for Bsep, thus providing structural support to the functional changes in bile salt secretion.

Although the mechanism(s) by which Bsep is internalized cannot be completely ascertained from our experiments, both our own results and those reported in the literature provide some hints. For example, (1) oxidizing compounds, like tBOOH and menadione, induce blebbing associated with actin cytoskeletal disarrangement in couplets, as reported in previous studies by our group (Ahmed-Choudhury et al., 1998; Stone et al., 1996), and confirmed by the results obtained here; (2) actin cytoskeleton disarrangement, like that induced by phalloidin, provokes internalization of the canalicular transporter, Mrp2 (Rost et al., 1999). It is therefore conceivable that, at least in part, Bsep internalization is causally related to F-actin disarrangement. Our results here indicate that this seems to be the case. A relationship between actin cytoskeleton and Bsep localization was to be anticipated, given that HAX-1, a cytoskeleton-associated protein that interacts with the F-actin–binding protein, cortactin, is a binding partner for canalicular Bsep (Ortiz et al., 2004), and that HAX-1 and cortactin participate in clathrin-mediated endocytic internalization of Bsep from the canalicular plasma membrane (Ortiz et al., 2004).

Whatever the mechanism of Bsep internalization, PKC activation seems to play a key role. Protein kinase C activation occurs in liver when a shift of the redox status toward moderate oxidative stress exists, as that induced by low concentrations of redox-cycling quinones (e.g., menadione and DMNQ) (Kass et al., 1989) or tBOOH (Kishimoto et al., 2000). In previous studies in couplets, we showed that PKC activation induced by vasopressin or phorbol esters promotes a number of morphological and functional hepatobiliary alterations in a Ca\textsuperscript{2+}-dependent manner, including actin disarray and bile salt secretory failure (Roma et al., 1998); this latter was further demonstrated in isolated rat perfused liver, and was attributed to Bsep internalization (Kubitz et al., 2003a). Furthermore, Kubitz et al. showed that Mrp2 localization and activity is impaired by PKC activation in HepG2 hepatoblastoma cells (Kubitz et al., 2001). These findings support the hypothesis that oxidative stress–induced alterations are mediated by activation of Ca\textsuperscript{2+}-dependent PKCs, and would be prevented, or even reversed, by PKC inhibition. The results obtained in this study using a wide range of PKC inhibitors and one Ca\textsuperscript{2+}-sequestering agent fully confirmed this contention.

Prevention/reversal of PKC-mediated disarrangement of the cytoskeleton may be a key protective mechanism against oxidative stress–induced bile salt secretory dysfunction. Protein kinase C phosphorylates and/or induces disorganization of several actin-cytoskeletal components, including actin itself, actin-associated proteins (e.g., α-actinin, vinculin, and filamin) and membrane-cytoskeletal cross-linked proteins, including Rac and myristoylated alanine-rich C-kinase substrate (MARCKS) (Keenan and Kelleher, 1998). Furthermore, PKC activates mitogen-activated protein kinases (MAPKs) in hepatocytes, acting as an alternative activator of Raf-1 (Romanelli and van de Werve, 1997), and MAPKs are involved in bile salt–induced apoptosis (Webster and Anwer, 1998), as well as in microfilament reorganization and alterations of cytoskeletal-membrane complexes following hypo-osmotic stress in hepatocytes (Kim et al., 2001). Whether the harmful effect of PKC involves preceding activation of phosphoinositide 3 kinase, which activates Ca\textsuperscript{2+}-dependent PKCs in hepatocytes (Carini et al., 2004) and has been implicated in the rearrangement of actin microfilaments in response to oxidative stress (Kang et al., 2002), is unknown.

Direct Bsep phosphorylation at sites involved in the crosslinking between actin and Bsep is also likely. The ATP-binding-cassette transporter Mdr1, the closest Bsep homolog, is phosphorylated by PKC at 3 serine residues within the “linker” region of the carrier, HAX-1 (Chambers et al., 1993)—i.e., the protein domain that interacts with the actin-associated protein. Whether this phosphorylation also occurs for Bsep, and whether these changes induce alterations in Bsep anchoring to the canalicular membrane, remains to be ascertained. Whatever the target, our demonstration that, under mild oxidative stress conditions, actin disarrangement can be fully prevented by PKC inhibitors complements the well-established view that oxidative stress induces crosslinking of cytoskeletal protein thiol groups by promoting the oxidation of the actin sulfhydryl group on a cysteine in position 374 (Dalle-Donne et al., 2001); the latter phenomenon may predominate only when high oxidative stress levels are operating. Cooperative actions of the direct oxidation of thiol groups and PKC-mediated phosphorylation occur for other proteins associated to the actin cytoskeleton, such as Keap1, which controls translocation of transcription factor Nrf2 to the nucleus, where it induces expression of several chemoprotective enzymes (Lee and Surh, 2005).

Because oxidative stress–induced bile salt secretory dysfunction and Bsep relocalization depended fully on PKC activation, direct oxidation of relevant structures involved in Bsep localization/function, or other indirect effects of ROS on cell integrity, can be ruled out as key causal mechanisms at low oxidative stress levels. For example, Schmitt et al. (2000) proposed that cell dehydration occurring under oxidative stress conditions may explain Mrp2 retrieval, because hyperosmolarity-induced hepatocellular shrinking relocates canalicular transporters. However, this is unlikely to apply here, however, because cellular shrinking induces this phenomenon via PKC-independent mechanisms (Schmitt et al., 2001).

Bile salt secretory failure induced by oxidative stress is not irreversible in nature, but it can be quickly reversed when oxidative stress–induced signaling misbalance is counteracted by using PKC inhibitors (see Fig. 5). Reversion of bile salt secretory failure was accompanied by extensive reduction of plasma membrane bleb formation, an early indicator of actin

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cytoskeleton disarray (data not shown), suggesting again a close relationship between bile salt secretory function and actin integrity. Protein phosphorylation status results from a balance between protein kinases and protein phosphatases. Therefore, a predominant role for the latter under conditions of PKC inhibition is likely to explain this fast reversion.

Our results also show that PKA activation with DB-cAMP was as effective as PKC inhibition in both preventing and reversing bile salt secretory dysfunction, and rearrangement of Bsep and F-actin. Although not conclusive, because of potential cross-reactivity with other kinases, the finding that the PKA inhibitor KT5720 abolished the beneficial action of DB-cAMP suggests that the effect is mediated by PKA activation. Cross-talk between PKC- and PKA-mediated signaling pathways exists and, often, they counterbalance each other to elicit regulated cellular responses (Yoshimasa et al., 1987). For example, several proteins involved in agonist-promoted PKC activation are inhibited by PKA, including GTP-binding proteins involved in coupling receptor function to phospholipase-C and phospholipase-C itself (Wen et al., 1992). Protein kinase A–induced effects on signaling pathways operating downstream of PKC can also be involved. For example, PKA activation inhibits PKC-induced MAPK activity in different cell lines by preventing Raf-1 activation by Ras (Liebmann, 2001). In hepatocytes, cAMP prevents bile salt–induced apoptosis (Webster and Anwer, 1998), an event involving both ROS formation (Patel and Gores, 1997) and PKC activation (Jones et al., 1997), by counteracting MAPK activation (Webster and Anwer, 1998).

Finally, direct beneficial effects of cAMP not mediated by transductional modifications of the PKC-dependent signaling pathway may exist. We have shown that cAMP stimulates F-actin cytoskeletal reassembly and recovery of bile salt secretory function after loss of couplet polarity following isolation (Roma et al., 2000); the latter event is likely to be due to cAMP stimulation of exocytic insertion of Bsep into its membrane domain (Kipp et al., 2001). It is therefore tempting to speculate that the protective effect of cAMP involves shift of the misbalance induced by oxidative stress on actin-cytoskeletal integrity and Bsep endocytosis, with stimulation of actin organization and exocytic Bsep insertion, factors that may well act in concert.

In conclusion, our results show that low oxidative stress levels impair bile salt secretory function by internalizing Bsep, through a Ca2+-dependent, PKC-mediated mechanism; alterations in actin organization seems to be a critical causal factor. These findings complement the common view that ROS impairs hepatobiliary transport by oxidizing structures relevant to bile formation by showing that, at low oxidative stress levels, ROS induce hepatocanicular damage, chiefly by evoking potentially deleterious signaling transductional cascades. Recognition that this relationship plays a key role should stimulate attempts to find complementary/alternative therapeutic strategies designed to control this signaling misbalance.

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