

Biliary Secretion of S-Nitrosogluthathione Is Involved in the Hypercholeresis Induced by Ursodeoxycholic Acid in the Normal Rat

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Ursodeoxycholic acid (UDCA) induces bicarbonate-rich hypercholeresis by incompletely defined mechanisms that involve the stimulation of adenosine triphosphate (ATP) release from cholangiocytes. As nitric oxide (NO) at a low concentration can stimulate a variety of secretory processes, we investigated whether this mediator could be implicated in the choleretic response to UDCA. Our *in vivo* experiments with the *in situ* perfused rat liver model in anesthetized rats, showed that UDCA infusion increased the biliary secretion of NO derivatives, hepatic inducible NO synthase expression, and NO synthase activity in liver tissue. UDCA also stimulated NO release by isolated rat hepatocytes. In contrast to UDCA, cholic acid was a poor inducer of NO secretion, and tauroursodeoxycholic acid showed no effect on NO secretion. Upon UDCA administration, NO was found in bile as low-molecular-weight nitrosothiols, of which S-nitrosogluthathione (GSNO) was the predominant species. UDCA-stimulated biliary NO secretion was abolished by the inhibition of inducible NO synthase with N_ω-nitro-L-arginine methyl ester in isolated perfused livers and also in rats whose livers were depleted of glutathione with buthionine sulfoximine. Moreover, the biliary secretion of NO species was significantly diminished in UDCA-infused transport mutant [ATP-binding cassette C2 (ABCC2)/multidrug resistance-associated protein 2 (Mrp2)-deficient] rats, and this finding was consistent with the involvement of the glutathione carrier ABCC2/Mrp2 in the canalicular transport of GSNO. It was particularly noteworthy that in cultured normal rat cholangiocytes, GSNO activated protein kinase B, protected against apoptosis, and enhanced UDCA-induced ATP release to the medium; this effect was blocked by phosphoinositide 3-kinase inhibition. Finally, retrograde GSNO infusion into the common bile duct increased bile flow and biliary bicarbonate secretion. **Conclusion:** UDCA induces biliary secretion of GSNO, which contributes to stimulating ductal secretion. (HEPATOLOGY 2010;52:667-677)

Abbreviations: ABC, adenosine triphosphate-binding cassette; AE2, anion exchanger 2; AKT, protein kinase B; ATP, adenosine triphosphate; BSO, buthionine sulfoximine; BV, beauvericin; BW, body weight; CA, cholic acid; GSNO, S-nitrosogluthathione; iNOS, inducible nitric oxide synthase; IPRL, isolated and perfused rat liver; isPRL, *in situ* perfused rat liver; L-NAME, N_ω-nitro-L-arginine methyl ester; LMw-SNO, low-molecular-weight nitrosothiol; LY294002, 2-morpholin-4-yl-8-phenylchromen-4-one; Mrp2, multidrug resistance-associated protein 2; MS, mass spectrometry; NO, nitric oxide; NOS, nitric oxide synthase; NRC, normal rat cholangiocyte; PI3K, phosphoinositide 3-kinase; SNO, nitrosothiol; TR⁻, transport mutant; TUDCA, tauroursodeoxycholic acid; UDCA, ursodeoxycholic acid; WT, wild type.

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Ursodeoxycholic acid (UDCA) is a hydrophilic bile acid widely used for the treatment of diverse cholangiopathies.¹ Despite its widespread clinical use, the mechanisms involved in the effects of UDCA are not yet completely understood. Its therapeutic potential appears to be related both to a change in the bile composition (with an increased concentration of hydrophilic bile salts versus hydrophobic bile salts) and to the stimulation of bicarbonate-rich choleresis.² Concerning the latter, it has been recently shown that UDCA stimulates apical adenosine triphosphate (ATP) release by cholangiocytes with further activation of purinergic receptors, elevation of the intracellular Ca^{2+} concentration, and stimulation of Cl^- efflux through Ca^{2+} -dependent Cl^- channels.³ Increased luminal Cl^- may be followed by $\text{Cl}^-/\text{HCO}_3^-$ exchange via anion exchanger 2 (AE2)⁴ with enhanced ductal bicarbonate and fluid secretion. However, the molecular mechanisms responsible for UDCA-induced hypercholeresis remain to be fully clarified.

Nitric oxide (NO) is a gaseous mediator of many biological functions and is synthesized from L-arginine by nitric oxide synthase (NOS) in a variety of tissue and cell types.⁵⁻⁷ NO is produced constitutively at low levels by endothelial and neuronal NOS and in variable proportions by inducible nitric oxide synthase (iNOS). In the liver, iNOS may be expressed by hepatocytes, cholangiocytes, hepatic stellate cells, and Kupffer cells.⁷ NO is able to exert dichotomous effects under physiological and pathological conditions.⁸ The induction of iNOS in phagocytic cells by a variety of noxious stimuli may lead to high and sustained levels of NO, which may cause cytotoxicity through nitrosative stress.⁹ At low or physiological concentrations, however, NO has been reported to defend cells from apoptosis^{10,11} and to modulate a vast variety of processes, including neurotransmission, relaxation of smooth muscle, and stimulation of different secretions such as bile flow and biliary glutathione secretion,⁶ intestinal Cl^- secretion, and pancreatic HCO_3^- secretion.^{7,12,13}

NO has a half-life of only 0.05 to 1.8 milliseconds.¹⁴ The major immediate breakdown product is nitrite (NO_2^-). This substance, like its nitrate derivative NO_3^- , is devoid of biological activity at physiological concentrations.¹⁵ Recent studies have shown that, once NO is generated, it is not merely degraded into these products but can be transported by thiol nitrosation of cysteinyl residues of proteins (especially albumin) and low-molecular-weight thiols, of which glutathione is the major NO transport species.¹⁶ In the

form of nitrosothiols (SNOs), the half-life of NO is prolonged, and it is able to act outside the site of synthesis,¹⁵ where it influences cellular signal transduction pathways and behaves as a critical modulator of many physiological processes. Here we show that the infusion of UDCA promotes hepatic synthesis and biliary secretion of S-nitrosoglutathione (GSNO). Biliary transport of this compound is partly mediated by the canalicular carrier ATP-binding cassette C2 (ABCC2)/multidrug resistance-associated protein 2 (Mrp2). GSNO activates protein kinase B (AKT) in cholangiocytes, protects against apoptosis, and enhances UDCA-induced ATP release to the lumen and thus contributes to stimulation of ductal secretion. These findings illustrate the fact that hepatocytes produce a mediator able to act downstream in the biliary tree and convey NO signals to cholangiocytes to enhance choleresis.

Materials and Methods

Animal Models. Male rats [body weight (BW) ~250 g] were employed for UDCA infusion studies according to either the *in situ* perfused rat liver (isPRL) model or the isolated and perfused rat liver (IPRL) model (see the supporting information). For the studies using the isPRL model in anesthetized rats, UDCA was administered through the femoral vein to (1) normal Wistar rats (40, 60, and 80 $\mu\text{mol}/\text{hour}$), (2) rats with depletion of liver glutathione after 2 days of treatment with buthionine sulfoximine (BSO; Sigma; UDCA at 80 $\mu\text{mol}/\text{hour}$), and (3) ABCC2/Mrp2-deficient [transport mutant (TR^-)] rats (UDCA at 80 $\mu\text{mol}/\text{hour}$). For specificity experiments, either cholic acid (CA) or tauroursodeoxycholic acid (TUDCA) was administered (80 $\mu\text{mol}/\text{hour}$ each) instead of UDCA. To assess the direct effect of GSNO on biliary epithelium, this compound was injected through the common bile duct of isPRLs. At the end of the experiments, blood was extracted from the portal vein, and animals were sacrificed, the liver and the common bile duct both being stored at -80°C until use. Inhibition of NO synthesis was assessed with the IPRL model by the infusion of UDCA in the presence or absence of the NOS inhibitor N_ω -nitro-L-arginine methyl ester (L-NAME; Sigma). Bile was collected throughout the different perfused liver experiments at 10-minute intervals. All animal studies were carried out in accordance with the *Guide for the Care and Use of Laboratory Animals* and were approved by the Ethical Committee for Animal Research of the University of Navarra.

Cell Cultures and ATP Determination. Hepatocytes were isolated from healthy male Wistar rats (~250 g) by collagenase perfusion as described.¹⁷ Cells with viability greater than 90% according to Trypan blue exclusion were seeded onto collagen-coated six-well plates (1×10^6 cells per well) and incubated at 37°C for 24 hours, and this was followed by treatment with 25 μ M UDCA in the presence or absence of 10 μ M cycloheximide. Supernatants were collected at 0, 15, 30, 60, and 90 minutes for the measurement of NO species.

Normal rat cholangiocytes (NRCs) were isolated and grown on rat-tail collagen with enriched Dulbecco's modified Eagle's medium/Ham's F-12 medium as described.^{4,18} Once cells reached confluence, the collagen layer was digested for 1 hour at 37°C with 0.66 mg/mL type XI collagenase (Sigma) and 1.66 mg/mL dispase (Gibco), and cells were washed with Dulbecco's phosphate-buffered saline. Cell monolayers were equilibrated in a 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid-based buffer for 1 hour at 37°C and treated for 5 minutes with 500 μ mol/L UDCA or CA,³ with 250 μ mol/L GSNO (synthesized as described¹⁹), and with a combination of UDCA (500 μ mol/L) and GSNO (250 μ mol/L). Media from different treatments were collected, and secreted ATP was measured with a commercial kit from Molecular Probes (Eugene, OR). Luminescence was quantified with an Infinite 200 microplate luminometer (Tecan, Männedorf, Switzerland). For phosphoinositide 3-kinase (PI3K) inhibition, 30 μ M 2-morpholin-4-yl-8-phenylchromen-4-one (LY294002; Sigma) was employed.

Determination of Bicarbonate, Nitrite/Nitrate, Glutathione, and NOS Activity. Bicarbonate was determined in bile with a Beckman Synchron CX3 analyzer (Beckman, Alvertville, MN). NO_2^- and NO_3^- in bile and cell supernatants were measured with a nitrate/nitrite colorimetric assay kit from Cayman Chemical (Ann Arbor, MI). The hepatic glutathione concentration was quantified with a commercial kit from Sigma, and the total NOS activity in liver tissue was measured with a radioactivity-based NOS activity assay kit from Cayman Chemical. The protein concentration in samples was determined according to Bradford's method.²⁰

Quantification of Biliary SNOs. Total SNOs and low-molecular-weight nitrosothiols (LMw-SNOs) were measured in bile with 4,5-diaminofluorescein (Calbiochem).²¹ This compound (10 μ L) was added to 1-mL diluted bile samples (200 μ L in phosphate-buffered saline with or without 0.2% HgCl_2). After 10 minutes of incubation at room temperature, fluorescence was

measured at an excitation wavelength of 495 nm and an emission wavelength of 515 nm. The SNO content (F_{SNO}) was estimated as the difference between the fluorescence measured in the presence of HgCl_2 (F_1) and the fluorescence measured in its absence (F_2 ; i.e., $F_{\text{SNO}} = F_1 - F_2$). In addition, 300- μ L bile samples were filtered with Centricon devices with a molecular weight cutoff of 10 kDa (Millipore, Billerica, MA), and the LMw-SNO content was measured in the filtrate as described previously.

Mass Spectrometry (MS) Analysis. To characterize biliary glutathione and GSNO, MS analysis was performed after protein precipitation via the mixing of a 500- μ L sample with an equal volume of 98% acetonitrile and 0.2% formic acid. After 30 minutes of incubation on ice, samples were centrifuged at 4000g. Supernatants were then infused with a 100- μ L syringe connected to a Q-TOF Micro instrument (Waters, Milford, MA) through a PicoTip nanospray ionization source (Waters). The heated capillary temperature was 80°C, and the spray voltage was 1.8 to 2.2 kV. MS data were collected and processed with Masslynx 4.1 (Waters).

Western Blotting. Homogenates from liver samples, common bile ducts, and NRCs were subjected to western blot analysis as described²² with antibodies against iNOS (Santa Cruz Biotechnology, Santa Cruz, CA), Akt, or phosphorylated Akt (Ser⁴⁷³; Cell Signaling, Beverly, MA). For a loading control, a β -actin antibody (Sigma) was employed.

Statistical Analysis. Results are expressed as means and standard errors of the mean. Comparisons of quantitative variables among groups were made with analysis of variance or Kruskal-Wallis tests (followed by the Student *t* test or Mann-Whitney nonparametric test, respectively), as required. A *P* value < 0.05 was considered to be significant.

Results

UDCA Increases Biliary NO Secretion. UDCA administration through the femoral vein in anesthetized rats with a cannulated common bile duct (i.e., the isPRL model) induced a dose-related increase in both the biliary total amount (Fig. 1A) and the concentration (data not shown) of the NO-breakdown products NO_2^- and NO_3^- , and this reflected increased biliary NO secretion (Fig. 1A). This effect was paralleled by the expected dose-dependent stimulation of choleresis and biliary bicarbonate output (Fig. 1A). Biliary NO secretion correlated significantly with both bile flow (Fig. 1A; *P* < 0.001) and biliary bicarbonate secretion (not shown). To assess the specificity of these

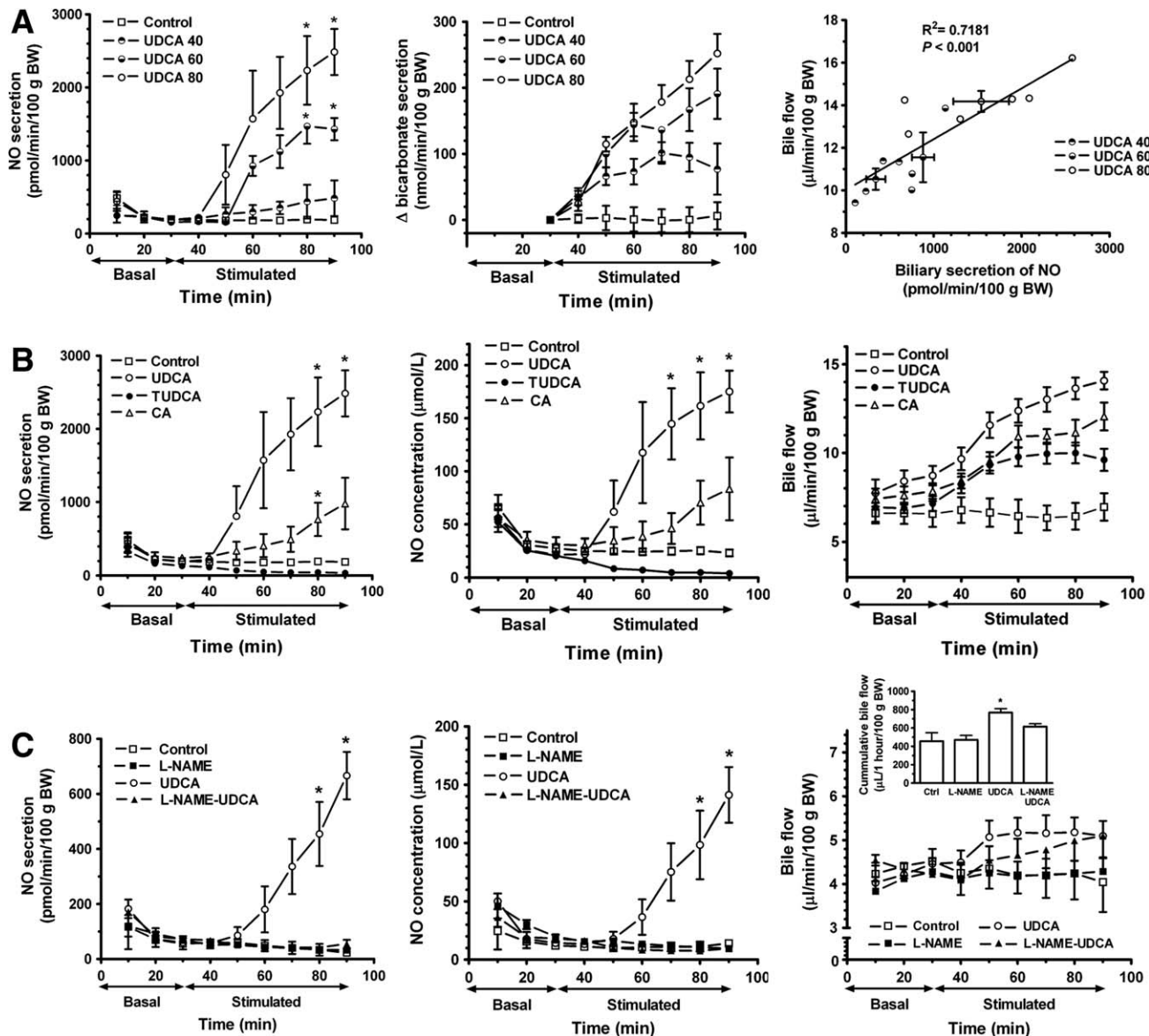


Fig. 1. UDCA stimulates biliary secretion of NO. (A,B) isPRL in anesthetized rats ($n = 3-7$). (C) IPRL ($n = 3$ or 4). In both perfused liver models (isPRL and IPRL), the secretion rate and concentration of NO (measured as nitrites/nitrates) were determined in bile fractions collected under basal conditions (30 minutes) and during stimulation with the perfusion of different bile salts from minute 30 to 90. (A) Dose-dependent effect of UDCA (infused at 40, 60, or 80 $\mu\text{mol}/\text{hour}$) on biliary NO secretion (left), bicarbonate secretion (middle), and the correlation between biliary NO secretion and choleresis (right); $*P < 0.05$ versus the control group (additionally, most UDCA-induced increases in bicarbonate secretion shown in the middle panel were highly significant, though asterisks are not represented for simplicity). (B) The effect of UDCA (80 $\mu\text{mol}/\text{hour}$) on biliary NO secretion (left) and NO concentration (middle) was quite specific because other bile salts such as CA and TUDCA (also infused at 80 $\mu\text{mol}/\text{hour}$) exerted little or no effect, respectively. Furthermore, UDCA produced a hypercholeric effect in comparison with CA or TUDCA (right); $*P < 0.05$ versus the control group. (C) The association of 30 μM L-NAME with UDCA (80 $\mu\text{mol}/\text{hour}$) in the IPRL model inhibited the UDCA-induced biliary NO secretion (left) and NO concentration (middle) and reduced bile flow (right). $*P < 0.05$ for UDCA versus L-NAME plus UDCA. The inset represents the cumulative bile flow for 1 hour upon stimulation with a UDCA infusion (or vehicle as the control) in the absence or presence of L-NAME.

effects, additional experiments were carried out with CA and TUDCA (two bile acids that display lower choleric activity than UDCA). As shown in Fig. 1B, biliary NO secretion was only weakly stimulated by CA, whereas it was not induced by TUDCA. In experiments carried out in the isolated liver (i.e., the IPRL model), we also observed an increase in the bili-

ary NO output in response to UDCA infusion (Fig. 1C), and this increase was abrogated by pretreatment with the NOS inhibitor L-NAME (Fig. 1C). Also, L-NAME caused a reduction in the UDCA-stimulated bile flow (Fig. 1C). These findings suggest a direct role of NOS in the UDCA-stimulated biliary output of NO and choleresis.

UDCA Stimulates iNOS Expression in Liver Cells. In agreement with our observations revealing an elevation of hepatic NO output upon UDCA infusion (as discussed previously), we found that both iNOS protein levels and NOS activity were significantly increased in the liver tissue of UDCA-perfused animals compared to controls (Fig. 2A,B). Moreover, the incubation of isolated rat hepatocytes with UDCA for 60 minutes prompted the release of NO species into the medium. This effect was abolished when protein synthesis was blocked with cycloheximide (Fig. 2C). Altogether, our results demonstrate that UDCA can act on liver cells by up-regulating iNOS expression and stimulating NO synthesis. These effects were distinctive of UDCA as no changes in hepatic iNOS expression or NOS activity were observed upon the infusion of other bile salts such as CA or TUDCA (Fig. 2A,B).

UDCA Enhances Biliary Secretion of SNOs. Because functionally active NO can be transported in biological fluids in the form of SNOs,¹⁵ we analyzed these compounds in bile after UDCA infusion in the isPRL model. In the baseline situation, the biliary total SNO output was 35.7 ± 4.5 pmol/minute/100 g of BW, which represented 13% of the total nitrite/nitrate output. Twenty-four percent of total SNOs corresponded to LMw-SNOs with a molecular weight less than 10 kDa. In UDCA-infused rats, however, total SNO output was three times higher, that is, 107.5 ± 13.8 pmol/minute/100 g of BW (Fig. 3A), and this accounted for about 40% of the total nitrite/nitrate output. The SNO elevation in UDCA-stimulated bile was mainly at the expense of LMw-SNOs, which represented 66% of the total SNOs (versus 24% in the basal situation; Fig. 3A). In contrast to UDCA, the infusion of CA, which only slightly elevated the total biliary NO secretion, did not significantly modify biliary secretion of SNOs (Supporting Fig. 1).

Among LMw-SNOs, GSNO is particularly relevant as a carrier for NO in different biological systems.²³⁻²⁵ Because glutathione is present in bile at a high concentration, it seems likely that biliary LMw-SNOs correspond mainly to GSNO. Using MS analysis, we could confirm the presence of authentic GSNO in bile. This compound, which corresponds to the 337.25 *m/z* band (Fig. 3B), exhibited a modest increment upon UDCA infusion. The instability of GSNO under MS conditions might explain why this band is not predominant in the spectrum. However, as shown in Fig. 3B, the relative intensity of a 319.24 *m/z* band (seemingly corresponding to dehydrated GSNO) was manifestly higher in UDCA-stimulated bile versus basal

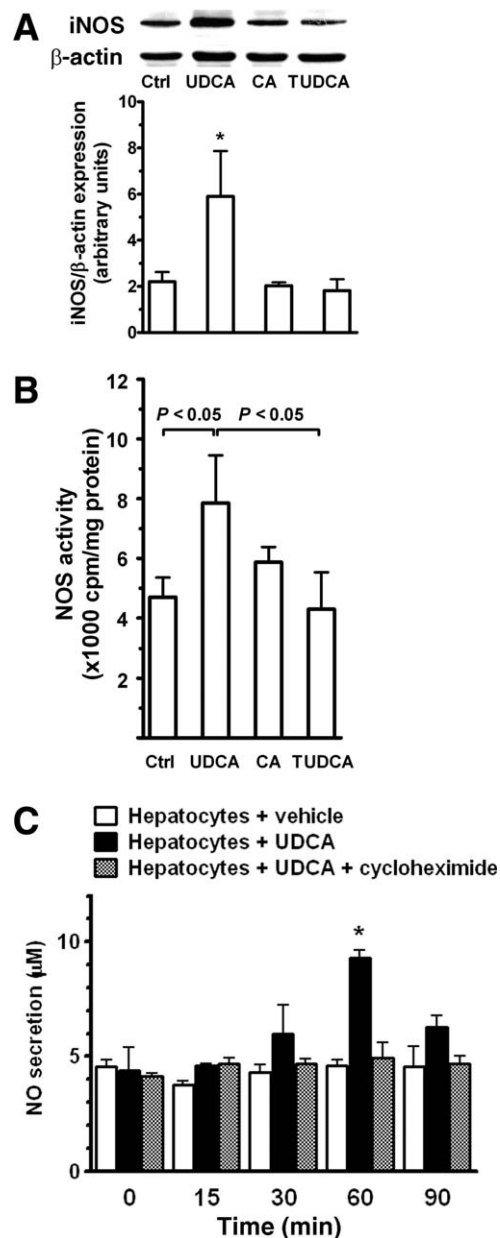


Fig. 2. UDCA induces iNOS protein expression and NOS activity in the liver. (A) Immunoblot showing iNOS protein expression in the liver tissue after the infusion of vehicle (control), UDCA, CA, or TUDCA in the isPRL model ($n = 4-6$). (B) The total NOS activity was measured in the same samples used in part A with a radioactivity-based kit (see the Materials and Methods section). (C) Secretion of NO from isolated rat hepatocytes incubated with 25 μ M UDCA in the presence or absence of 10 μ M cycloheximide. The total nitrites/nitrates were measured in cell supernatants collected at different incubation times (four independent experiments). * $P < 0.05$ versus the other groups.

bile. These data support the concept that UDCA infusion induces an increase of GSNO in bile.

We also assessed the involvement of glutathione in the transport of NO to bile by determining biliary NO in rats after depleting their livers of glutathione with BSO. As we previously reported,²⁶ UDCA

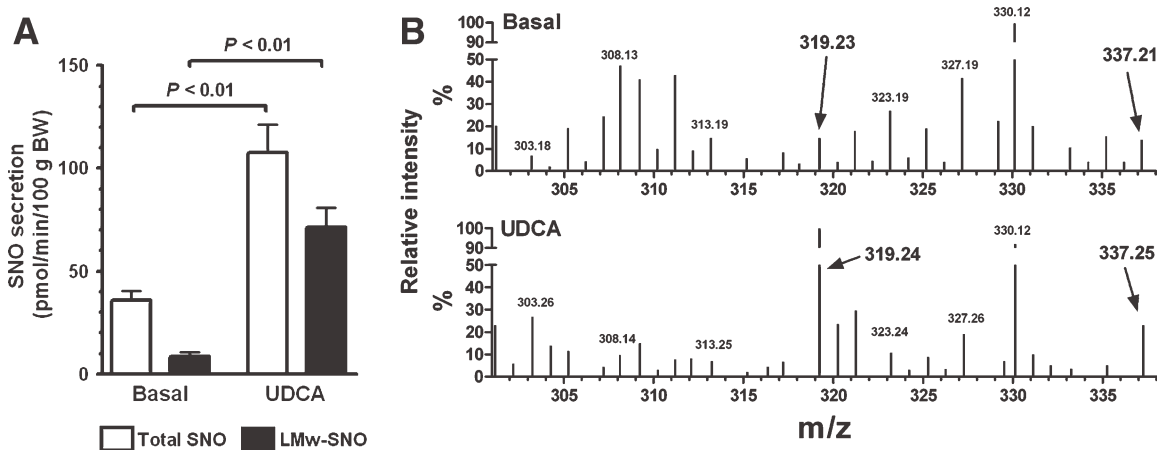


Fig. 3. Effect of UDCA on the biliary secretion of SNOs. (A) The total SNOs (white bars) and LMw-SNOs (<10 kDa; black bars) were measured in bile aliquots collected before (basal) and after the administration of UDCA (80 μ mol/hour) for 60 minutes ($n = 3$). (B) Typical MS spectra of biliary glutathione derivatives showing ions with m/z values of 337.2 (GSNO) and 319.2 (dehydrated GSNO). For MS analysis, bile samples collected for part A were previously deproteinized.

increased hepatic glutathione levels in normal rats (Fig. 4A). However, in rats that received BSO, liver glutathione was markedly reduced, regardless of UDCA administration (Fig. 4A). An analysis of UDCA in bile from UDCA-infused normal rats and BSO-treated rats showed that biliary UDCA secretion was similar in both situations (Supporting Fig. 2), and this indicates that the secretion of UDCA to bile is not prevented in the absence of glutathione. In contrast, the secretion of NO species after UDCA infusion does depend on glutathione, as it was virtually abolished in BSO-treated animals (Fig. 4B), even though their hepatic NOS activity was increased to levels similar to those found in UDCA-infused normal rats (data not shown). These findings are consistent with the notion that glutathione has a major role as a carrier for the transport of NO to bile.

ABCC2/Mrp2 Is Involved in Canalicular GSNO Secretion. Glutathione and glutathione conjugates are known to be secreted at the canaliculi through the ABCC/Mrp2 pump. Therefore, we performed UDCA infusion experiments in TR⁻ rats, which exhibit defective canalicular transport of those compounds because of an ABCC2 mutation.²⁷ In these animals, the levels of biliary glutathione fall 3 logs with respect to normal values, but the compound is still secreted to bile in the micromolar range.²⁸ As shown in Fig. 5A,B, UDCA-infused TR⁻ rats exhibited a significant decrease in both the concentration and biliary output of NO species in comparison with UDCA-infused normal rats. The increment in biliary NO secretion upon UDCA infusion in TR⁻ rats was less than half of that observed in normal animals ($P < 0.05$; see the inset in Fig. 5B). In the mutant rats, the levels of both total

SNOs and LMw-SNOs increased after UDCA administration, but the values were about one-third of those observed in UDCA-treated normal rats (Supporting Fig. 3). These findings indicate that the glutathione carrier ABCC2/Mrp2 contributes at least partially to biliary NO secretion and provide further support for a role of glutathione as a vehicle for the transport of NO along the biliary tree.

Retrograde Biliary Infusion of GSNO Increases Cholereresis. To determine whether GSNO could play a role in stimulating ductal secretion *in vivo*, we performed a retrograde infusion of 150 μ L of 250 μ M GSNO through the common bile duct in the isPRL model. As shown in Fig. 6, such a maneuver induced a significant increase in both bile flow and biliary bicarbonate secretion, and this confirmed the choleretic properties of GSNO.

GSNO Is Involved in ATP Release Through Akt Activation in Cultured NRCs. Previous studies have demonstrated that NO can stimulate ATP release from astrocytes.²⁹ In cholangiocytes, ATP release has been shown to participate in UDCA-induced hypercholereresis.³ To characterize the choleretic properties of GSNO, we analyzed whether this compound, alone or together with UDCA, could enhance ATP release from cultured NRCs. As shown in Fig. 7A, incubation of NRCs with 250 μ M GSNO resulted in a modest but not significant increase in ATP release to the medium in comparison with control values, whereas UDCA (500 μ M) significantly increased ATP release to the extracellular milieu. CA (500 μ M), however, failed to induce extrusion of ATP from cholangiocytes (Supporting Fig. 4). Interestingly, simultaneous incubation with both GSNO (250 μ M) and UDCA (500 μ M)

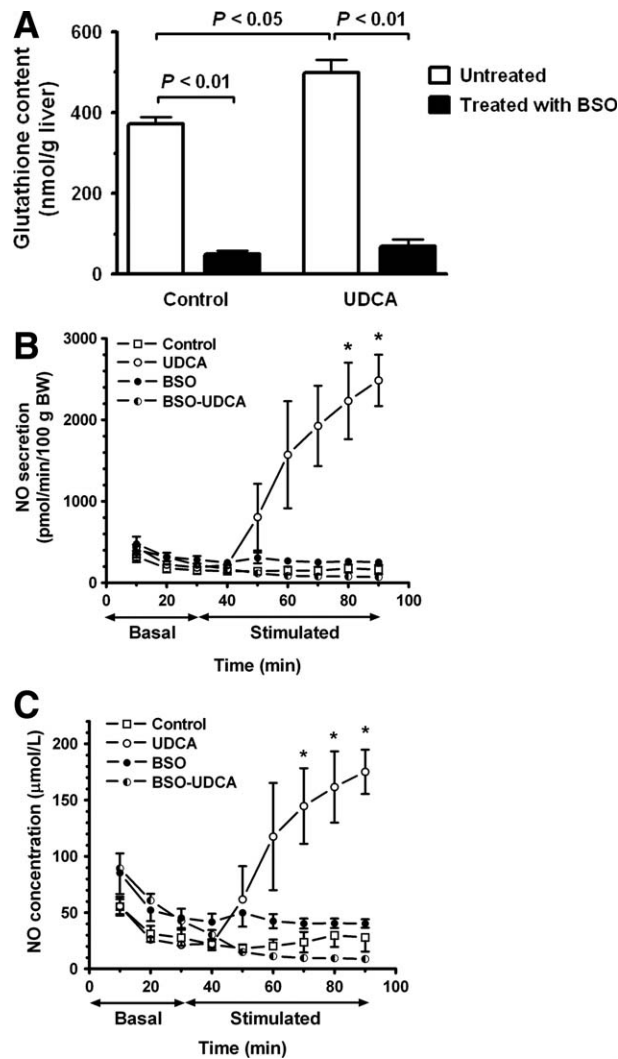


Fig. 4. Effect of liver glutathione depletion on the biliary secretion of NO. (A) The glutathione concentration was measured in the livers from untreated or BSO-treated rats after *in situ* perfusion under anesthesia and infusion with vehicle or UDCA (80 μmol/hour) from minute 30 to 90. (B) Secretion rate and (C) concentration of NO determined in the bile from anesthetized rats with and without a UDCA infusion as in part A. * $P < 0.05$ versus the remaining experimental groups.

caused significantly higher ATP release in comparison with 500 μM UDCA alone ($P < 0.05$; Fig. 7A). This finding supports the notion that GSNO may convey secretory signals to the bile duct epithelium contributing to the stimulation of ductal secretion induced by UDCA.

In cholangiocytes, UDCA has been shown to activate the PI3K/Akt signaling pathway.³⁰ This route has been recently implicated in secretory functions and vesicular trafficking,³¹ a process seemingly responsible for ductal ATP secretion. By using the PI3K inhibitor LY294002, we therefore analyzed whether this route could be involved in ATP release from NRCs in response to UDCA with and without GSNO. We

found that the inhibitor could abolish ATP release in all cases (Fig. 7A) and that GSNO alone, UDCA alone, and the combination of GSNO and UDCA were each able to phosphorylate AKT (Fig. 7B). In contrast, CA did not show AKT phosphorylating activity (Supporting Fig. 5). These observations demonstrate the ability of GSNO to activate AKT in NRCs and suggest that PI3K/AKT activation participates in ATP secretion by cholangiocytes. Because the AKT signaling pathway activates cell survival mechanisms,³² we analyzed whether GSNO is able to protect cultured NRCs against proapoptotic insults. In experiments employing beauvericin (BV)-treated NRCs, we found that the presence of GSNO was associated with a

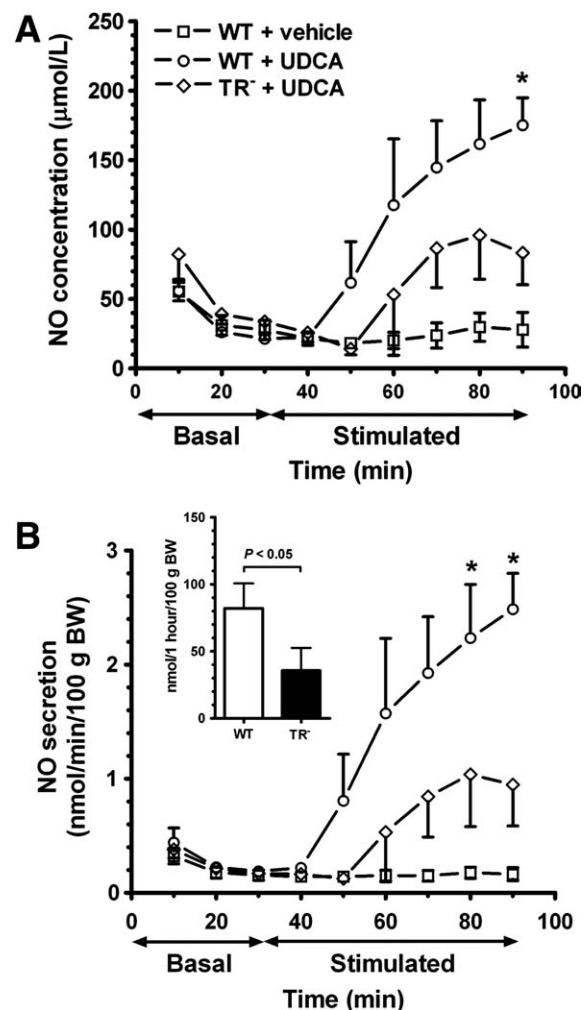


Fig. 5. Involvement of ABCG2/Mrp2 in the biliary secretion of NO. (A) Concentration and (B) secretion rate of NO determined in the bile from WT rats infused with just vehicle ($n = 7$, control), WT rats infused from minute 30 to 90 with UDCA at 80 μmol/hour ($n = 4$), and TR⁻ rats similarly infused with UDCA ($n = 4$). * $P < 0.05$ versus the same period in the other experimental groups. The inset in part B represents the cumulative amount of NO secreted to bile from minute 30 to 90 upon stimulation with a UDCA infusion in WT and TR⁻ rats.

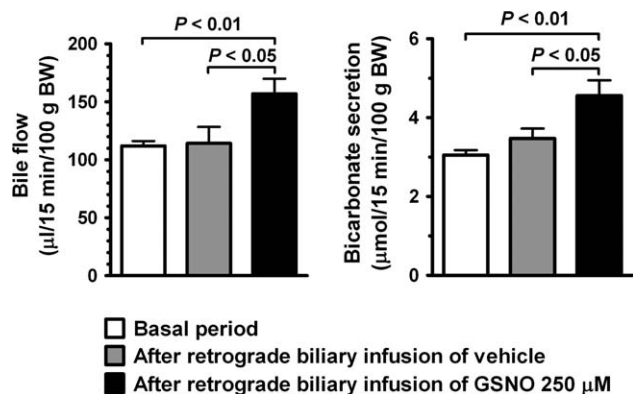


Fig. 6. Effect of a retrograde biliary infusion with GSNO on the bile flow (left) and biliary bicarbonate secretion (right) in anesthetized rats. These parameters were determined in bile fractions collected before (basal period) and after a retrograde intrabiliary infusion with 150 μ L of either vehicle or 250 μ M GSNO ($n = 5$ for each) through the common bile duct of the isPRL model.

reduction in cell death, which reached statistical significance when the NO donor was used at 500 μ M (Fig. 7C).

Discussion

In this study, we show that UDCA infusion through the femoral vein of normal rats increases biliary secretion of NO-derived molecular species in a dose-dependent manner. The elevated output of NO species is due to up-regulated expression of iNOS and the subsequent rise in NOS activity in the liver. This effect on iNOS expression and biliary NO secretion appears to be distinctive for UDCA because it is not shared by other bile acids such as CA and TUDCA.

The notion that *ex novo* NO synthesis is involved in the response to UDCA is supported by our data from the isolated liver model, in which the burst of UDCA-induced biliary NO output was completely abolished by the NOS inhibitor L-NAME. Moreover, experiments carried out with freshly isolated rat hepatocytes indicated that these cells can release NO species when they are incubated with UDCA. UDCA-induced release of NO from isolated hepatocytes was not observed in the presence of the protein synthesis inhibitor cycloheximide, and this supports the involvement of increased production of iNOS in these liver cells.

It has been shown that SNOs prolong the NO half-life and allow this molecule to be transported in biological fluids to fulfill biological functions at places distant from the point at which it is produced.¹⁵ The considerable abundance of glutathione in bile makes this compound an excellent carrier for the transport of functional NO along the biliary tree. In our study, we

observed a great rise in SNOs upon UDCA infusion, mainly at the expense of SNOs with a molecular weight less than 10 kDa, and this strongly suggests that GSNO is the most likely NO carrier in bile. MS analysis confirmed that, upon UDCA infusion, there was a rise in GSNO and putative GSNO derivatives in bile. Moreover, when the rat liver was depleted of glutathione with BSO, the infusion of UDCA failed to induce any increase in biliary NO, and this further reinforces the view that GSNO has an essential role as the NO carrier in bile.

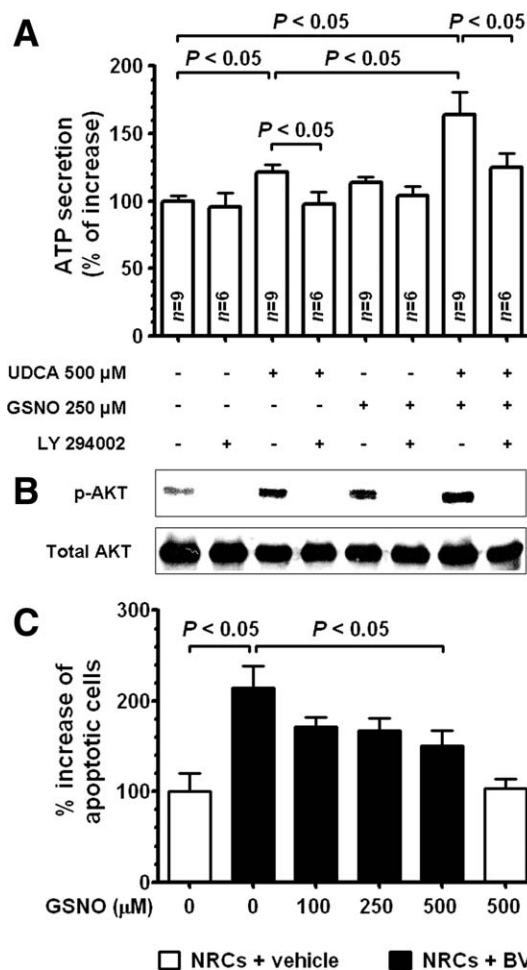


Fig. 7. ATP release, AKT activation, and antiapoptotic effect in NRCs incubated with GSNO. (A) ATP secretion from NRCs incubated for 5 minutes with 500 μ M UDCA, 250 μ M GSNO, or a combination of the two. The stimulated release of ATP was blocked in the presence of the PI3K antagonist LY294002. The values of ATP luminescence are represented as percentage increases with respect to ATP values for cells incubated with vehicle. (B) Immunoblot detection of phosphorylated AKT and total AKT in protein extracts from NRCs (treated as previously indicated). Images are representative of four independent immunoblots. (C) Measurement of apoptotic cells after the treatment of NRCs with 25 μ M BV in the presence or absence of different concentrations of GSNO. Values are percentage increases of apoptotic cells with respect to those measured in vehicle-treated NRCs (six independent experiments).

ABCC2/Mrp2 is a canalicular protein involved in the transport of glutathione and glutathione conjugates to bile (reviewed by Ballatori et al.³³). In TR⁻ rats, which have defective ABCC2 function,²⁷ there is a marked impairment in biliary secretion of glutathione, the concentration of which falls from the normal millimolar range to a micromolar range.²⁸ This impairment is associated with decreased biliary NO secretion in response to UDCA. The level of total biliary NO (which is normally excreted at concentrations in the micromolar range) falls to less than half of normal values, and the level of biliary SNOs falls to about one-third of normal. This observation reveals a role of ABCC2 in mediating, at least in part, the canalicular efflux of NO species from hepatocytes to bile. Furthermore, this finding is consistent with the existence of a link between biliary NO secretion and biliary transport of glutathione.

Although GSNO might be the prevalent NO species in canalicular bile, its catabolism or degradation to nitrites/nitrates within the bile ducts may create a gradient of decreasing concentration along the biliary tree. Also, a portion of the GSNO that remains intact in the bile duct lumen may enter the bile duct epithelial cells. It thus seems probable that the actual concentration of GSNO that is secreted at the canaliculi and drains into the intrahepatic bile ducts is substantially higher than that detected at the end of the common bile duct.

Our data indicate that the secretion of GSNO to bile is critical for the hypercholeretic activity of UDCA. Indeed, UDCA-stimulated bile flow in the IPRL decreased in the presence of L-NAME. The observed flow reduction corresponded to the fraction accounting for the hypercholeretic effect of UDCA. The notion that luminal GSNO can activate ductal secretion is strengthened by our *in vivo* studies showing that retrograde infusion of GSNO through the common bile duct of rats causes a significant increase in both bile flow and biliary bicarbonate secretion. Supporting the view that UDCA-induced secretion of NO derivatives to bile might be important for UDCA-induced bicarbonate-rich hypercholeresis, a previous study has shown that the administration of exogenous SNOs such as *S*-nitroso-*N*-acetylpenicillamine increases choleresis and biliary secretion of HCO₃⁻ in IPRL.⁶ This study certainly uncovered the choleretic properties of infused SNOs, although it provided no information regarding the influence of UDCA on hepatobiliary NO metabolism or the role of endogenously formed GSNO in UDCA-induced hypercholeresis.

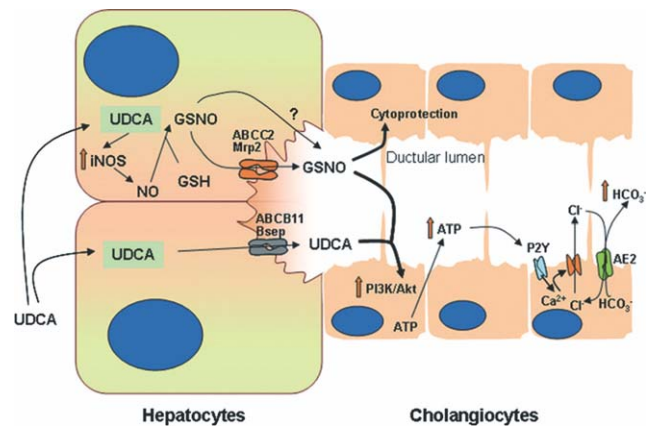


Fig. 8. Proposed mechanism for UDCA-induced hypercholeresis. UDCA induces iNOS and increases NO production in hepatocytes. Once combined with glutathione, NO is secreted into bile in the form of GSNO. Biliary secretion of GSNO occurs, at least in part, via the canalicular glutathione carrier ABCC2/Mrp2. There is also a possibility that part of the NO newly synthesized in the hepatocyte exits through the canaliculus by alternative mechanisms. In cholangiocytes, GSNO induces AKT activation and, in conjunction with UDCA, promotes the release of ATP to the lumen. Luminal ATP is known to activate P2Y purinergic receptors, and this leads to elevation of cytosolic Ca²⁺ and activation of Ca²⁺-dependent Cl⁻ channels, which is followed by AE2-mediated Cl⁻/HCO₃⁻ exchange and increased bicarbonate-rich choleresis.

In order to gain insight into how GSNO might stimulate ductal secretion, we investigated the effect of GSNO on the release of ATP by NRC. Recently, it has been shown that ATP release to the ductal lumen and subsequent activation of purinergic receptors on the luminal membrane of cholangiocytes are key initial events in the choleretic response to UDCA.^{3,34} We have found that GSNO is able to markedly increase UDCA-induced ATP release by cholangiocytes. It seems likely that this effect of GSNO contributes to the choleretic properties of UDCA. These findings are in agreement with studies of other tissues showing that NO can induce ATP release in astrocytes and resensitize purinergic receptors in mesangial cells in rats^{35,36} and thus linking NO metabolism and signaling through ATP. On the other hand, it has been shown that the PI3K/AKT signaling cascade plays a key role in exocytosis and ATP release in cholangiocytes.³⁷ Accordingly, we have found that GSNO can efficiently activate AKT and that PI3K blockade with LY294002 prevents the strong release of ATP induced by GSNO when it is given together with UDCA.

As mentioned previously in the introduction, NO may have dichotomous effects. High levels of NO generated by phagocytic cells in response to proinflammatory cytokines may compromise cell function by causing nitrosative stress. Under these conditions, NO may impair cyclic adenosine monophosphate-mediated

cholangiocyte secretion leading to NO-mediated cholestasis.³⁸ However, under physiological conditions, NO may behave as a cytoprotective molecule that is also able to stimulate diverse biological activities, including secretory functions. In fact, we found that GSNO, in addition to stimulating ATP release, was able to protect cultured NRCs against BV-induced apoptosis. This is consistent with the ability of GSNO to activate the PI3K/AKT signaling pathway, a route known to promote both cell survival and secretory functions.^{31,32}

In summary, our findings indicate that an infusion of UDCA may activate iNOS in liver cells and thus promote the canalicular secretion of NO species, which mainly occurs in the form of GSNO. Once transported to bile at least in part through ABCC2/Mrp2, GSNO can stimulate secretory functions in bile ducts by inducing activation of the PI3K/AKT signaling pathway and the release of ATP (Fig. 8). These observations have identified endogenous GSNO as a molecule able to activate secretory and cytoprotective functions in cholangiocytes. Our study has also revealed a new mechanism for the therapeutic properties of UDCA, a bile acid benefiting patients with chronic cholangiopathies¹ by stimulating ductal bile formation and defending cholangiocytes against injury.³⁹

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