Ursodeoxycholic acid and superoxide anion

Predrag Ljubuncic, Omar Abu-Salah, Arieh Bomzon

INTRODUCTION

Ursodeoxycholic acid (UDCA) is a naturally occurring tertiary dihydroxy hydrophilic bile acid used with considerable success in the treatment of primary biliary cirrhosis (PBC)\[7,8\]. Traditional mechanisms whereby UDCA is beneficial to the diseased liver center on its ability to block the deleterious actions and encourage the choleresis of toxic bile acids\[8\]. We have reported that UDCA has antioxidant properties. This finding is based on our observation that 100 µmol/L UDCA could prevent oxidative activation of cultured macrophages by the pro-oxidant hydrophobic bile acid, deoxycholic acid\[13,14\]. In a follow-up in vivo study, we demonstrated that the therapeutic dose of 15 mg/kg UDCA administered for 24 d suppresses the augmented extent of lipid peroxidation in the diseased liver of bile duct-ligated (BDL) rats, a widely used animal model of cholestatic liver disease\[9\]. Accordingly, we proposed the antioxidant action of UDCA could contribute to its beneficial effect in patients with PBC\[7,8\]. This proposal was recently confirmed by Serviddio and his colleagues who reported UDCA administered for 28 d minimized oxidative damage in the diseased liver of BDL rats\[9\]. Other studies have established that UDCA can augment the biosynthesis of glutathione (GSH) and thiol-containing proteins in hepatocytes\[10,11\] and could scaveng the hydroxyl radical (OH\(^-\)) in a cell-free system\[12\]. Collectively, these findings have contributed to our current awareness that UDCA is a binary antioxidant possessing direct and indirect antioxidant properties.

Superoxide anion (O\(^2-\)) is a reactive oxygen species (ROS) generated in microsomal and mitochondrial electron transport systems when oxygen is reduced by a single electron. It can also be generated by numerous oxidative enzymes including cytosolic xanthine oxidase (XO) during oxidation of xanthine (X) to uric acid. The generation of O\(^2-\) is important, because its biotransformation can lead to formation of hydrogen peroxide through the activity of superoxide dismutase, generation of OH\(^-\) radical in the presence of transition metals such as Fe\(^2+\) or formation of the reactive peroxynitrite radical by interacting with nitric oxide. Any intervention reducing or preventing excessive generation of O\(^2-\) would result in decreased production of reactive oxygen and nitroxy species thereby lowering the extent of oxidative stress.

Mitsuyoshi et al\[16\], reported that UDCA increases hepatocyte levels of GSH and thiol-containing proteins. In this experiment, they spectrophotometrically measured the rate of oxidation of ascorbic acid (AA) by O\(^2-\) and reported that the difference of absorbance before and after the addition of XO was lower in UDCA-treated hepatocytes than in controls. Since bile acids are enzyme inhibitors\[15,16\], the difference in absorbance may be due to UDCA inhibiting the ability of the X-XO system to generate O\(^2-\). Because Mitsuyoshi et al., did not assess the effect of UDCA on the generation of O\(^2-\) by X-XO system in their investigation, we evaluated the ability of UDCA to scavenge O\(^2-\) generated in the X-XO system and its effect on the activity of XO.

MATERIALS AND METHODS

Chemicals and reagents

All chemicals and reagents of the highest purity were

AIM: To investigate the ability of ursodeoxycholic acid (UDCA) to scavenge superoxide anion (O\(^2-\)).

METHODS: We assessed the ability of UDCA to scavenge O\(^2-\) generated by xanthine-xanthine oxidase (X-XO) in a cell-free system and its effect on the rate of O\(^2-\)-induced ascorbic acid (AA) oxidation in hepatic post-mitochondrial supernatants.

RESULTS: UDCA at a concentration as high as 1 mmol/L did not impair the ability of the X-XO system to generate O\(^2-\), but could scavenge O\(^2\) at concentrations of 0.5 and 1 mmol/L, and decrease the rate of AA oxidation at a concentration of 100 µmol/L.

CONCLUSION: UDCA can scavenge O\(^2-\), an action that may be beneficial to patients with primary biliary cirrhosis.

© 2005 The WJG Press and Elsevier Inc. All rights reserved.

Key words: Ursodeoxycholic acid; Superoxide anion; Antioxidant

Ljubuncic P, Abu-Salach O, Bomzon A. Ursodeoxycholic acid and superoxide anion. World J Gastroenterol 2005; 11(31): 4875-4878

http://www.wjgnet.com/1007-9327/11/4875.asp

World J Gastroenterol 2005;11(31):4875-4878
World Journal of Gastroenterology ISSN 1007-9327 © 2005 The WJG Press and Elsevier Inc. All rights reserved.
purchased from the Sigma Chemical Co. (St. Louis, MO, USA), except for UDCA that was purchased as its sodium salt from Megapharm Ltd, the Israeli agent of Calbiochem-Novabiochem Corporation.

**Preparation of hepatic post-mitochondrial supernatants**
The livers were harvested from healthy rats used as “healthy untreated controls” in institute-approved animal-based investigations. The harvested livers were washed in normal ice-cold saline, weighed and then cut into small pieces using scissors before their homogenization in 100 mmol/L ice-cold phosphate buffer, pH 7.4 at 4 °C with a Potter-Elvehjem glass homogenizer. The crude liver homogenates were centrifuged (1 000 g/min×10 min) at 4 °C. The resultant supernatants were then centrifuged at 10 000 g×10 min at 4 °C to pellet mitochondria and the supernatant was collected. The protein content in the supernatants was determined by the method of Lowry et al.[18].

**Analytical procedures**
**Ability of UDCA to scavenge superoxide anion** Using the xanthine-xanthine oxidase (X-XO) reaction to generate O$_2^-$[19], we evaluated the ability of 0.01–1 mmol/L UDCA to scavenge O$_2^-$ by the nitroblue tetrazolium (NBT) reduction assay[17]. The reaction mixture contained 100 µmol/L Na$_2$EDTA, 40 µmol/L X, and 40 µmol/L NBT in 10 mmol/L phosphate buffer pH 7.4. The reaction was started by adding 10 µm/L XO and its rate was continuously monitored spectrophotometrically at $A_{560}$ nm for 15 min at 25 °C in the absence and presence of different concentrations of UDCA. The specificity of the reaction was confirmed by 300 µm/mL superoxide dismutase. The ability of UDCA to scavenge O$_2^-$, as expressed as percentage of inhibition of NBT reduction in UDCA-present samples compared to NBT reduction in UDCA-absent samples. The experiment was repeated between 9 and 11 times at each UDCA concentration.

**Effect of UDCA on activity of xanthine oxidase**
Compounds interacting with XO could affect the kinetics of reaction of oxidation of xanthine to uric acid[18]. Accordingly, we assessed the effect of 0.01–1 mmol/L UDCA on XO activity by spectrophotometrically monitoring the rate of uric acid formation at $A_{295}$ nm for 3 min at 25 °C in the absence and presence of UDCA[19]. The rate of uric acid formation was compared in the absence and presence of UDCA. The specificity of the reaction was confirmed by 100 µg/mL allopurinol. The experiment was repeated nine times at each UDCA concentration.

**Effect of UDCA on hepatic antioxidant capacity**
The effect of 100 µmol/L UDCA on hepatic antioxidant capacity was determined by monitoring the rate of oxidation of AA by the O$_2^-$ generated by the X-XO according to the original method of Nishikimi[24] with modifications described by Mitsuyoshi et al.[10]. Liver supernatants were incubated for 120 min at 37 °C in shaking water bath in the absence and presence of 100 µmol/L UDCA. Upon completion of the incubation, the change in the rate of oxidation of AA was monitored in a quartz cuvette containing 1 mL reaction mixture. The post-mitochondrial liver supernatant contained 100 µmol/L X, 100 µmol/L EDTA, 22 µg/mL catalase, 100 µmol/L AA and 0.1 mol/L phosphate buffer pH 7.4. The assay reaction was commenced by adding 10 µm/mL XO. The reaction was monitored spectrophotometrically at $A_{290}$ nm for 10 min at 25 °C. The differences in absorbance between UDCA-absent and UDCA-added samples were compared. The results were expressed as the reaction rate of AA oxidation ($\Delta$ absorbance/mg protein). The experiment was repeated thrice.

**Experimental design and statistical analysis**
The data were analyzed using a two-tailed t-test. The sample size for each experiment was determined by power analysis arbitrarily set between 80–90% in order to detect the effect at the 5% significance level using StatmateTM version 1 (GraphPad Software Inc., San Diego, CA, USA). All figures were prepared using PrismTM version 3.02 (GraphPad Software Inc., San Diego, CA, USA). All data were expressed as mean±SD.

**RESULTS**
**Ability of UDCA to scavenge O$_2^-$ and effect of UDCA on activity of xanthine oxidase**
Figure 1A summarizes the experiments to establish whether UDCA could scavenge O$_2^-$ using the NBT reduction assay. UDCA (10 µmol/L and 100 µmol/L) had no effect on NBT reduction (Figures 1A1 and A2). Higher concentrations of UDCA (500 µmol/L and 1 mmol/L) slowed the rate of NBT reduction by 11% and 16% respectively (Figure 1A3 and A4). In order to eliminate the possibility that UDCA suppressed the rate of conversion of X to uric acid to account for this O$_2^-$ scavenging ability, we also measured the effects of the identical concentrations of UDCA on XO activity. None of the UDCA concentrations affected the rate of formation of uric acid (Figure 1B). Overall, these experiments demonstrated UDCA could scavenge O$_2^-$ without affecting the activity of XO.

**Effect of UDCA on rate of oxidation of ascorbic acid**
UDCA (100 µmol/L) significantly (P<0.002) decreased the rate of AA oxidation (Figure 2).

**DISCUSSION**
The aim of the present study was to assess the ability of UDCA to scavenge O$_2^-$ and its effects on the activity of XO in a cell-free system and post-mitochondrial supernatants prepared from rat livers. In the cell-free experiments, we used UDCA concentrations not greater than 1 mmol/L in order to avoid the problems when extrapolating data to cell-containing systems in which millimolar concentrations of bile acids solubilize membranes to form micellar poly-aggregates (critical micellar concentration)25-28. On the other hand, we used 100 µmol/L UDCA in the experiments conducted in post-mitochondrial supernatants because this is the concentration used by Mitsuyoshi et al.[10], and that found in the plasma of patients treated with UDCA[22]. Using NBT reduction in the cell-free X-XO system to evaluate the ability of UDCA to scavenge O$_2^-$, we found UDCA
could scavenge $O_2^-$ at concentrations of 0.5 and 1 mmol/L, respectively. Given the knowledge that bile acids can inhibit enzyme activity\cite{13,14}, we checked the possibility that suppression of the activity of XO accounted for ability of UDCA to scavenge $O_2^-$. We found that UDCA at concentrations as high as 1 mmol/L did not inhibit the rate of conversion of X to uric acid. After establishing that UDCA could not impair the ability of the X-XO system to generate $O_2^-$, we then evaluated the effect of 100 $\mu$mol/L UDCA on the rate of $O_2^-$-induced AA oxidation in hepatic post-mitochondrial supernatants. We found that UDCA decreased the rate of AA oxidation. This result agrees with that of Mitsuyoshi et al\cite{10}.

Is the scavenging of $O_2^-$ by UDCA therapeutically relevant? Free-radical-induced peroxidation of phospholipids has been implicated in the pathogenesis of the formation of cholesterol gallstones\cite{25-27}. In addition, Liu and Hu demonstrated that $O_2^-$ can attack the bilirubin molecule to generate cytotoxic bilirubin radicals. ROS can also be generated in bile because hydrophobic bile acids are pro-oxidants\cite{28,29}. It was reported that the concentration of UDCA in bile may reach as high as 29 mmol/L in individuals given 750 mg UDCA daily for 2-3 wk\cite{30}. In our experiments, UDCA scavenged $O_2^-$ at the concentrations of 0.5 and 1 mmol/L suggesting that the ability of UDCA to scavenge $O_2^-$ may be beneficial in bile.

Lapenna et al\cite{12}, reported that UDCA is an efficient OH· scavenger. We have confirmed their finding and established the second order rate constant ($k_2$) for the reaction of UDCA with OH· in the D-ribose oxidation assay exceeded the rate constants of other OH· radical scavengers, such as mannitol or dimethylsulfoxide (Ljubuncic, Abu-Salach and Bomzon, unpublished results). In conclusion, UDCA is a direct scavenger of superoxide anion and hydroxyl radicals.

Figure 1 Ability of UDCA to scavenge $O_2^-$ (A) and effect of UDCA on activity of xanthine oxidase (B) in a cell-free system. *$P<0.02$, **$P<0.01$ vs control.

Figure 2 Effect of 100 $\mu$mol/L UDCA on rate of oxidation of AA by $O_2^-$ generated by X-XO in post-mitochondrial hepatic supernatants. *$P<0.002$ vs control.

REFERENCES

1. Poupon R, Chretien Y, Poupon RE, Ballet F, Calmus Y, Darnus F. Is ursodeoxycholic acid an effective treatment for primary biliary cirrhosis. *Lancet* 1987; 1: 834-836
2. Oka H, Toda G, Ikeda Y. A multi-center double-blind controlled trial of ursodeoxycholic acid for primary biliary cirrhosis. *Gastroenterol Jpn* 1990; 25: 774-780
3. Heathcote EJ, Cauch-Dudek K, Walker V, Bailey RJ, Blendis LM, Ghent CN, Michieletti P, Minuk GY, Pappas SC, Scully LJ, Steinbrecher UP, Sutherland LR, Williams CN, Witt-Sullivan H, Worobetz LJ, Milner RA, Wanless IR. The Canadian multicenter double-blind randomized controlled trial of ursodeoxycholic acid for primary biliary cirrhosis. *Hepatology* 1994; 19: 1149-1156
4. Lindor KD, Dickson ER, Baldus WP, Jorgensen RA, Ludwig J, Murtaugh PA, Harrison JM, Wiesner RH, Anderson ML, Lange SM, LeSage G, Rossi SS, Hofmann AF. Ursodeoxycholic acid in the treatment of primary biliary cirrhosis. *Gastroenterology* 1994; 106: 1284-1290
5. Leuschner U, Guldutina S, Imhof M, Hubner K, Benjamino
A, Leuschner M. Effects of ursodeoxycholic acid after 4 to 12 years of therapy in early and late stages of primary biliary cirrhosis. J Hepatol 1994; 21: 624-633

6 Paumgartner G, Beuers U. Ursodeoxycholic acid in cholestatic liver disease. mechanisms of action and therapeutic use revisited. Hepatology 2002; 36: 525-531

7 Ljubuncic P, Fuhrman B, Oknine J, Aviram M, Bomzon A. Effect of deoxycholic acid and ursodiolocholic acid on lipid peroxidation in cultured macrophages. Gut 1996; 39: 475-478

8 Ljubuncic P, Tanne Z, Bomzon A. Ursodeoxycholic acid suppresses extent of lipid peroxidation in diseased liver in experimental cholestatic liver disease.Dig Dis Sci 2000; 45: 1921-1928

9 Serviddio G, Pereda J, Pallardo FV, Carretero J, Borras C, Cutrin J, Vendemiale G, Poli G, Vina J, Sastre J. Ursodeoxycholic acid protects against secondary biliary cirrhosis in rats by preventing mitochondrial oxidative stress. Hepatology 2004; 39: 711-720

10 Mitsuyoshi H, Nakashima T, Sumida Y, Yoh T, Nakajima Y, Ishikawa H, Inaba K, Sakamoto Y, Okano T, Kashima K. Ursodeoxycholic acid protects hepatocytes against oxidative injury via induction of antioxidants. Biochem Biophys Res Comm 1999; 263: 537-542

11 Rodriguez-Ortigosa CM, Cincu RN, Sanz S, Ruiz F, Quiroga J, Prieto J. Effect of ursodeoxycholic acid on methionine adenosyltransferase activity and hepatic glutathione metabolism in rats. Gut 2002; 50: 701-706

12 Lapenna D, Ciofani G, Festi D, Neri M, Pidovmeci SD, Giamberardino MA, Cuccurullo F. Antioxidant properties of ursodeoxycholic acid. Biochem Pharmacol 2002; 64: 1661-1667

13 Parkinson TM, Olson JA. Inhibitory effects of bile acids on adenosine triphosphatase, oxygen consumption, and the transport and diffusion of water soluble substances in the small intestine of the rat. Life Sci 1964; 3: 107-112

14 Krakenbuhl S, Stucki J, Reichen J. Reduced activity of the electron transport chain in liver mitochondria isolated from rats with secondary biliary cirrhosis. Hepatology 1992; 15: 1160-1166

15 Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folkin phenol reagent. J Biol Chem 1951; 193: 265-275

16 McCord JM, Fridovich I. The reduction of cytochrome c by milk xanthine oxidase. J Biol Chem 1968; 243: 5753-5760

17 Aruoma OI, Halliwell B, Hoey BM, Butler J. The antioxidant action of N-acetylcysteine: its reaction with hydrogen peroxide, hydroxyl radical, superoxide, and hypochlorous acid. Free Radic Biol Med 1989; 6: 593-597

18 Fridovich I. Quantitative aspects of the production of superoxide anion radical by milk xanthine oxidase. J Biol Chem 1970; 245: 4053-4057

19 Marcocci L, Suzuki YJ, Tsujiya M, Packer L. Antioxidant activity of nitecapone and its analog OR-1246: effect of structural modification on antioxidant action. Methods Enzymol 1994; 234: 525-541

20 Nishikimi M. Oxidation of ascorbic acid with superoxide anion generated by the xanthine-xanthine oxidase system. Biochem Biophys Res Comm 1975; 63: 463-468

21 Heaton KW. The importance of keeping bile salts in their place. Gut 1969; 10: 857-863

22 Heuman DM. Quantitative estimation of the hydrophilic-hydrophobic balance of mixed bile salt solutions. J Lipid Res 1989; 30: 719-730

23 Roda A, Minutello A, Angellotti MA, Fini A. Bile acid structure-activity relationship: evaluation of bile acid lipophilicity using 1-octanol/water partition coefficient and reverse phase HPLC. J Lipid Res 1990; 31: 1433-1444

24 Steihl A, Rudolph G, Raedsch R, Moller B, Hopf U, Lotterer E, Bircher J, Folsch U, Klaus J, Enende R, Senn M. Ursodeoxycholic acid-induced changes of plasma and urinary bile acids in patients with primary biliary cirrhosis. Hepatology 1990; 12: 492-497

25 Lichtenberg D, Ragimova S, Peled Y, Halpern Z. Phospholipid peroxidation as a factor in gallstone pathogenesis. FEBS Lett 1988; 228: 179-181

26 Eder MI, Miquel JF, Jungst D, Paumgartner G, Von Ritter C. Reactive oxygen metabolites promote cholesterol crystal formation in model bile: Role of lipid peroxidation. Free Radic Biol Med 1996; 20: 743-749

27 Leo MA, Aleyink SI, Siegel JH, Kasmin FE, Aleyink MK, Lieber CS. F2-isoprostane and 4-hydroxynonenal excretion in human bile of patients with biliary tract and pancreatic disorders. Am J Gastoenterol 1997; 92: 2069-2072

28 Sokol RJ, Devereaux M, Khandwala R, O’Brien K. Evidence for involvement of oxygen free radicals in bile acid toxicity to isolated rat hepatocytes. Hepatology 1993; 17: 869-881

29 Sokol RJ, Winkloheer-Roob BM, Devereaux MW, McKim JM. Generation of hydroperoxides in isolated rat hepatocytes and hepatic mitochondria exposed to hydrophobic bile acids. Gastroenterology 1995; 109: 1249-1256

30 Fischer S, Neubrand M, Paumgartner G. Biotransformation of orally administered ursodeoxycholic acid in man as observed in gallbladder bile, serum and urine. Eur J Clin Invest 1993; 23: 28-36

Science Editor Wang XL and Guo SY  Language Editor Elsevier HK