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INHIBITION OF BILIARY EXCRETION OF INDOCYANINE GREEN BY THE THIOL-OXIDIZING AGENT, DIAZENEDICARBOXYLIC ACID BIS[*N,N'*-DIMETHYLAMIDE]

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Abstract—Biliary excretion of Indocyanine green (ICG) in Sprague–Dawley rats during constant intravenous infusion of the dye *in vivo* was inhibited by intraperitoneally administered diazenedicarboxylic acid bis[*N,N'*-dimethylamide] (diamide, 0.5 mmol/kg body wt), a glutathione-specific thiol-oxidizing agent. Significant inhibition of ICG excretion was observed also when ICG was injected rapidly 90 min after diamide administration. Disappearance of ICG from the plasma was not affected by diamide. Oxidized glutathione in bile increased transiently following diamide administration but returned to the basal level within 30 min. Hepatic concentrations of reduced and oxidized glutathione were not different from those of controls when determined 90 min after diamide administration. The inhibition of ICG excretion was completely prevented by subsequent administration of dithiothreitol (0.5 mmol/kg) 30 min after that of diamide. The results, therefore, suggest that the biliary excretion of ICG was inhibited by secondary changes in the redox status of thiols in hepatocytes caused by a transient increase in oxidized glutathione.

Indocyamnine green (ICG†) is carried in the plasma bound to lipoproteins [1, 2] and is extracted exclusively by the liver, to be excreted into bile without metabolic modifications [3]. Several proteins on the sinusoidal membranes of hepatocytes have been indicated to be transporters of several organic anions [4–6], and they may also transport ICG across the membrane. However, little is known about the mechanism of biliary excretion of the dye. We have found that a thiol-oxidizing agent, diamide [7–9], markedly inhibits the biliary excretion of ICG in rats when administered i.p., and in this study the mechanism of this inhibition was investigated.

MATERIALS AND METHODS

Diamide and acivicin were purchased from the Sigma Chemical Co. (St Louis, MO, U.S.A.). ICG was purchased from the Daiichi Seiyaku Co. (Tokyo, Japan). Other chemicals used were of analytical grade. Sprague–Dawley rats (weighing 300–350 g), fed *ad lib.* on conventional rat food, were used in all experiments.

The common bile duct was cannulated with PE-10 polyethylene tubing while the rat was anesthetized with pentobarbital (50 mg/kg body wt). The bile volume was measured gravimetrically using 1 as the specific gravity. ICG concentration was determined spectrophotometrically by absorbance at 805 nm.

Constant infusion of ICG. ICG was dissolved in distilled water (18 mg/mL) and diluted with 4 vol. of physiological saline. The solution was infused via PE-50 tubing cannulated into the jugular vein at a constant rate of 0.05 mL/min/kg body wt (i.e. 180 μg ICG/min/kg body wt). The bile was collected at 10-min intervals. After 60 min of infusion, the biliary excretion rate of ICG approached a plateau and diamide (0.5 mmol/kg dissolved in mL/kg physiological saline) was administered i.p. Changes in bile flow and ICG excretion were observed for the next 90 min while ICG was infused at the same rate.

Effect of diamide on glutathione in the bile and liver. Since the reduced form of glutathione (GSH) is rapidly auto-oxidized to its oxidized form (GSSG) in untreated bile [10], the effects of diamide on GSH and GSSG in the bile were examined in another series of experiments. An irreversible γ-glutamyltranspeptidase inhibitor, acivicin (20 μmol/kg in 0.4 mL/kg distilled water), was administered retrogradely into the bile duct to inhibit intraductular hydrolysis of glutathione [11, 12]. Bile was collected in tubes, each containing 0.2 mL of 5% metaphosphoric acid, to inhibit oxidation of GSH [10]. Diamide (0.5 mmol/kg i.p.) was administered and the bile was collected for the next 90 min. At the end of each experiment, the liver was excised and homogenized with phosphate-buffered 0.25 mol/L sucrose (pH 7.4). The homogenate (25% w/v) was mixed with a volume of 10% trichloroacetic acid and the deproteinized supernatant was obtained by centrifugation [13].

Glutathione concentration in bile and liver was determined by the method of Tietze [13] as total glutathione (GSH + GSSG). GSSG concentration was determined independently (as GS equivalent)

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† Abbreviations: ICG, Indocyanine green; diamide, diazenedicarboxylic acid bis[*N,N'*-dimethylamide]; GSH, reduced glutathione; GSSG, oxidized glutathione.

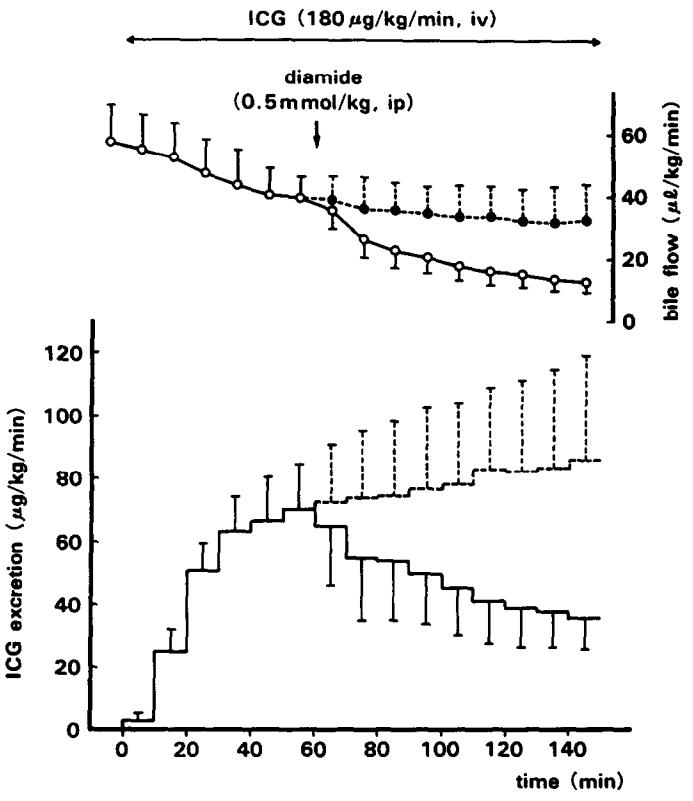

Fig. 1. Effect of diamide on bile flow rate and ICG excretion. ICG was infused at a rate of 180 μg/kg/min from $t = 0$ to $t = 150$. Diamide (0.5 mmol/kg, i.p.) was administered at $t = 60$. Bile was collected at 10-min intervals. Top: bile flow rates of diamide-treated rats (solid line, N = 4) and controls (broken line, N = 4). Bottom: biliary excretion rates of ICG of diamide-treated rats (solid line) and controls (broken line). Vertical lines indicate 1 SD.

by the method of Griffith [14] using 4-vinylpyridine to mask GSH at the final concentration of 0.5% (v/v). GSH concentration was calculated as the difference between the two values.

Bolus injection of ICG. Bolus ICG injection studies were performed to determine the duration and reversibility of the effect of diamide. ICG was dissolved in distilled water (5 mg/kg in 1 mL/kg) and injected rapidly into the jugular vein. Rats were divided into four groups. In groups A and B, rats were pretreated with diamide (0.5 mmol/kg i.p.) 90 min prior to the ICG injection. In group B, dithiothreitol (0.5 mmol/kg in mL/kg physiological saline, i.p.) was also administered 60 min prior to the ICG injection. Rats in group C received dithiothreitol only; those in group D, physiological saline instead of diamide. Bile was collected for 90 min after ICG injection.

Plasma disappearance of ICG. In several experiments with bolus ICG injection, blood (0.3 mL each) was drawn from the femoral vein 5, 10, and 15 min after the injection of ICG. The serum was separated by centrifugation and the concentration of ICG was measured. The time-course of the disappearance of ICG from the plasma was revealed to be monoexponential, and the exponential regression was calculated using the least-squares method.

RESULTS

In the constant ICG infusion studies, biliary excretion rates of ICG began to decrease 5 min after the administration of diamide and remained lower than the maximum level throughout the observation period (Fig. 1). Bile flow rates also decreased following diamide administration. Biliary excretion rates of GSSG increased during the first 20 min after diamide administration but returned subsequently to basal level (Fig. 2). Changes in the excretion rate of GSH were not significant. GSH and GSSG concentrations in the liver determined 90 min after diamide administration did not differ from control levels (Table 1).

The biliary excretion rates of ICG after bolus injection were lower in group A than in group D (controls) throughout the observation period (Fig. 3). However, ICG excretion rates did not decrease in group B. Cumulative ICG excretion after 90 min was 44% of the administered dose in rats in group A, while that in group D was 73% (Fig. 4). Rats in group A demonstrated a marked decrease in bile flow rate following diamide administration, with the maximum decrease between 15 and 20 min after administration (Fig. 5). ICG concentration in the bile was lower in group A (but not in group B) than

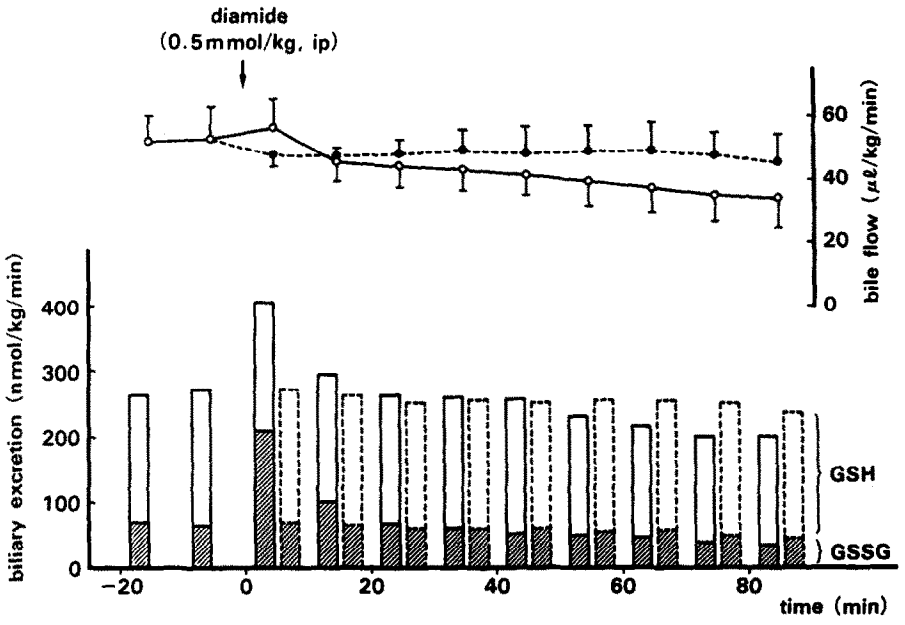

Fig. 2. Effect of diamide on biliary excretion of glutathione. Diamide (0.5 mmol/kg, i.p.) was administered at $t = 0$. Bile was collected at 10-min intervals. Biliary excretion rates of GSH (open bars) and GSSG (hatched bars) are shown. Values are means of four experiments with diamide-treated rats (bars with solid line) and controls (bars with broken line). One standard deviation was less than 10% of corresponding mean values. Changes in bile flow rate are shown (solid line, diamide-treated; broken line, controls; values are means and SD).

Table 1. Hepatic concentration of GSH and GSSG

	GSH (μmol/g liver)	GSSG (μmol/g liver)
Diamide-treated rats	4.98 ± 0.51	0.08 ± 0.06
Controls	4.62 ± 0.29	0.11 ± 0.08

Hepatic concentration of GSH and GSSG. Diamide (0.5 mmol/kg) was administered i.p. 90 min prior to the preparation of liver homogenate. Values are means ± SD, N = 4.

in group D between 5 and 30 min after ICG injection (Fig. 6). The disappearance of ICG from the plasma in group A was not different to that in group D (Table 2).

DISCUSSION

The time-course of the disappearance of ICG from the plasma was not altered by diamide treatment, indicating that diamide inhibited the biliary excretion of ICG from the liver without affecting the hepatic extraction of the dye. Diamide oxidizes GSH to GSSG chemically (*vide infra*); GSSG produced in the cell is avidly reduced to GSH by the cellular GSSG reductase–NADPH system and excessive GSSG, if any, is actively excreted into the bile [15–17]. GSSG in the bile had returned to its normal level 30 min after diamide administration, and the hepatic levels of GSH and GSSG determined 90 min after diamide treatment were not different from those of controls. This indicates that the oxidizing effect of diamide on GSH was almost completely eliminated 90 min after the administration of diamide. Nevertheless, ICG excretion was still significantly lower in diamide-treated rats than in controls when injected at that time. Thus, cellular GSSG increased by diamide is unlikely to have directly affected ICG excretion.

Diamide oxidizes not only GSH but also several other natural electron donors *in vitro* [8]. However, the kinetic studies by Kosower *et al.* [9] demonstrated that diamide reacts with GSH much more rapidly than it does with other substances, indicating that the conversion of GSH to GSSG is the most important immediate consequence of diamide treatment. On the other hand, protein thiol–disulfide status in the cell, known to be an important determinant of enzyme function and cell homeostasis, may be disturbed by changes in the redox status of glutathione through thiol–disulfide exchanges catalysed by thiol-transferase [18]. The effect of diamide on ICG excretion was completely antagonized by the subsequent administration of dithiothreitol, a non-selective thiol-reducing agent, 30 min after diamide, when GSSG in the bile had already returned to its basal level, suggesting that diamide affected not only the redox status of cellular glutathione but also that of other thiols. We, therefore, conclude that ICG excretion was inhibited by secondary changes in the redox status of cellular

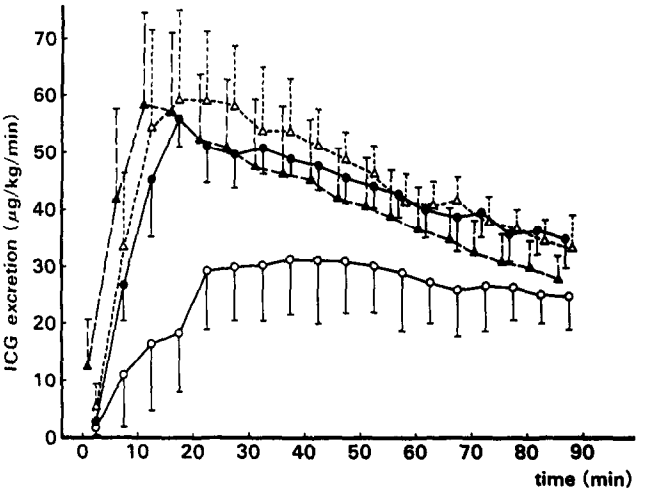

Fig. 3. Biliary excretion rate of ICG after bolus injection. ICG (5 mg/kg) was rapidly injected via the jugular vein at $t = 0$. Bile was collected at 5-min intervals. (A) (open circles, N = 6): pretreated with diamide (0.5 mmol/kg i.p. at $t = -90$); (B) (open triangles, N = 6): pretreated with both diamide (same as in A) and dithiothreitol (0.5 mmol/kg i.p. at $t = -60$); (C) (closed triangles, N = 4): pretreated with dithiothreitol only; and (D) (closed circles, N = 4): received physiological saline. Values are means and SD.

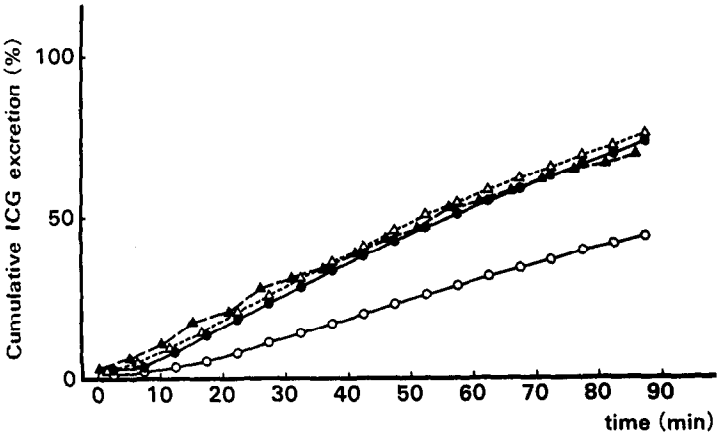

Fig. 4. Cumulative ICG excretion after bolus injection. For experimental protocol and symbols, see legend to Fig. 3. Values are the means of cumulative biliary excretion as percentages of injected dose.

thiols other than glutathione due to the transient increase in GSSG. Those thiols in the hepatocytes appeared to be essential for the biliary excretion of ICG. Akerboom *et al.* [19] reported the inhibition of biliary excretion of taurocholate by diamide and other agents as occurring by similar mechanisms.

Although ICG is relatively lipophilic, it is unlikely that ICG crosses plasma membranes by simple diffusion, as suggested by the selective uptake by hepatocytes. Biliary excretion of several organic anions such as bilirubin glucuronides, sulfobromophthalein and GSSG is impaired in two mutant rat strains [20–22]. An ATP-dependent anion transporter on the canalicular membrane has been indicated as being responsible for the canalicular transport of these substances and is defective in the mutants [23, 24]. It is possible that the transporter has affinity also for ICG, another organic anion, and is involved in the biliary excretion of the dye. However, the effect of diamide on the biliary excretion of the sulfobromophthalein–glutathione conjugate or bilirubin is transient and disappears when GSSG in the bile returns to the normal level (unpublished observations). Thus, the inhibition of ICG excretion by diamide was not caused by changes in the putative canalicular transporter for multiple organic anions.

The inhibition of ICG excretion by diamide was associated with marked decreases in bile flow rate, whether diamide was administered during ICG

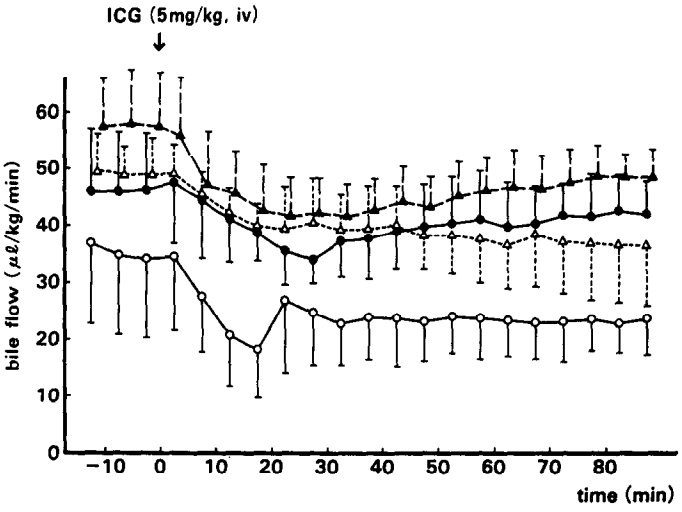

Fig. 5. Changes in bile flow rate after bolus injection of ICG. For experimental protocol and symbols, see legend to Fig. 3. Values are means and SD.

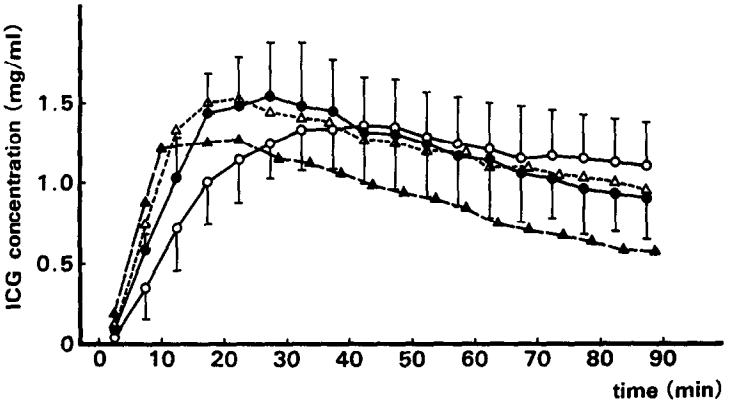

Fig. 6. ICG concentration in bile after bolus injection. For experimental protocol and symbols, see legend to Fig. 3. Values are mean and SD (not for triangles).

infusion or 90 min prior to the bolus injection of ICG. Thus, it is possible that diamide altered the composition of bile, predisposing ICG to precipitate in segments of the biliary tree and causing cholestasis

Table 2. Disappearance of ICG from plasma

	C_0 (mg/mL)	$T_{1/2}$ (min)
Diamide-treated rats	0.247 (0.220–0.278)	4.89 (4.12–6.01)
Controls	0.255 (0.211–0.309)	4.64 (4.09–5.36)

Monoexponential regression was calculated from the serum concentration of ICG 5, 10, and 15 min after bolus injection (5 mg/kg) assuming $C = C_0 \exp(-t \ln 2/T_{1/2})$, where C is the ICG concentration at time t and C_0 is the apparent initial concentration. Diamide (0.5 mmol/kg) was administered i.p. 90 min prior to the ICG injection. Ranges in parentheses are those of means ± 1 SD, N = 4.

outside the hepatocytes. The partitioning to biliary lipid particles is different between ICG and more hydrophilic organic anions [25]. Thus, diamide may selectively inhibit ICG excretion by causing cholestasis in the biliary tree if the lipid composition of bile is altered by diamide so that the solubility of ICG only is affected.

However, ICG at high hepatic concentrations is itself anti-choleretic [26–28] without affecting bile salt excretion [29] and toxic effects of ICG on mitochondrial functions *in vitro* have been reported [30]. Thus, ICG accumulated in hepatocytes will decrease bile formation. As shown in Fig. 5, the initial decrease of bile flow following ICG injection was partially reversed by 25 min when the ICG concentration in the hepatocytes was presumably lower than it was initially but was still increasing in the bile as shown in Fig. 6. It is, therefore, rather unlikely that the precipitation of ICG in the biliary tree played a major role in decreasing ICG excretion. Unfortunately no technique is currently available to

determine ICG levels in hepatocytes and bile canaliculi separately, and the exact site of ICG retention remains to be established.

In conclusion, we have demonstrated that the GSH-specific thiol-oxidizing agent, diamide, inhibits biliary excretion of ICG while not altering the hepatic extraction of the dye. The inhibitory effect of diamide continues for longer than changes in glutathione redox status in the bile and liver. The results suggest that not the transient increase in cellular GSSG by diamide administration but secondary changes in the redox status of thiols in hepatocytes inhibit the biliary excretion of ICG.

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