Effects of Duration of Ischemia and Donor Pretreatment with Methylprednisolone or Its Macromolecular Prodrug on the Disposition of Indocyanine Green in Cold-Preserved Rat Livers

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Purpose. Cold preservation of the liver before transplantation may change uptake and excretory functions of hepatocytes. We hypothesized that an increase in the duration of preservation would result in a progressive decrease in the hepatic uptake and/or biliary excretion of indocyanine green (ICG), which would be attenuated by pharmacologic interventions.

Methods. Donor rats (n = 40) were administered saline (control) or single 5 mg/kg doses of methylprednisolone (MP) or its liver-targeted prodrug (DMP) 2 h prior to liver harvest. Following preservation in cold University of Wisconsin solution for 0, 24, 48, or 72 h, livers were reperfused in a single-pass manner for 30 min in the presence of ICG (~4 μg/ml), followed by 60 min of ICG-free perfusion. The inlet, outlet, and bile concentrations of ICG were measured periodically by high performance liquid chromatography (HPLC), and kinetic parameters were estimated.

Results. Effects of duration of preservation: In unpreserved livers, a significant portion of ICG dose (16%) was effluxed from the liver during the washout period. Cold preservation for 24–72 h progressively increased ($p < 0.05$) the efflux of ICG (>2-fold at 72 h). Similarly, average extraction ratio showed a modest (30–40%) decrease with increasing preservation time ($p < 0.05$). However, biliary excretion of ICG showed the most sensitivity to the preservation time (14 to >800-fold decline). Effects of pretreatment: DMP caused significant ($p < 0.05$) increases in biliary ICG levels (>12-fold) and bile flow rates (6–15-fold) of preserved livers. Although MP pretreatment significantly ($p < 0.05$) increased (6-fold) bile flow rates in 48-h preserved livers, its effects on biliary ICG levels were not significant ($p > 0.05$).

Conclusions. Biliary excretion of ICG is the most sensitive kinetic parameter to prolonged cold ischemia-reperfusion injury in a rat liver perfusion model. The injury may be significantly attenuated by pharmacologic pretreatment of the liver donors.

KEY WORDS: dextran prodrugs; indocyanine green; ischemia-reperfusion; isolated perfused rat liver; methylprednisolone.

INTRODUCTION

In recent years, liver transplantation has become a well-established procedure for therapy of fatal liver diseases (1). In spite of dramatic improvements in this procedure and its outcome, preservation injury, occurring during cold ischemia and subsequent reperfusion (I/R), is still considered to be a crucial determinant of primary graft failure following liver transplantation (2). Considering the cell types involved, hepatocytes and biliary epithelia (3), in addition to non-parenchymal cells (4), may play a crucial role in the pathophysiology of preservation-reperfusion injury. Because metabolism and biliary elimination of drugs are mediated by hepatocellular uptake and/or excretory pathways (5), changes in these processes following I/R could impact the disposition of many xenobiotics.

Indocyanine green (ICG), a sulfonic acid dye, is widely used as a marker of graft function following clinical liver transplantation (6,7). Hepatic uptake and canalicular excretion of ICG is mediated by the organic anion transporting protein 1 (Oatp1) (8) and the multidrug resistance P-glycoprotein 2 (Mdr2) (9), respectively. Because ICG is not metabolized and is excreted unchanged into bile, it has been proposed (10) as a suitable marker for the study of hepatocellular uptake and biliary excretion processes.

Previous studies (4,7,11) have shown that the duration of preservation is one of the major determinants of graft function after liver transplantation; preservation times of >12 h have generally been associated with a substantial graft failure rate (7). This seriously limits the availability of the precious graft. Therefore, it is necessary to determine the effects of duration of preservation on the injury to different hepatic cells and devise methods to prevent or attenuate them.

Although non-parenchymal cells are extensively studied with regard to their role in I/R injury (4), the effects of preservation-induced I/R on hepatocyte functions have not received much attention. This is mainly because morphological damages to hepatocytes appear after significantly longer preservation times, compared with earlier damages observed with Kupffer cells (KCs) and endothelial cells (3,4). However, functional damages to hepatocytes may precede the apparent morphological changes (3). Therefore, a goal of the current study was to investigate the effects of duration of cold preservation on the hepatocyte functions using ICG as a marker in an isolated perfused rat liver (IPRL) model. Furthermore, we recently showed (12) that pretreatment of donor rats, before liver harvest, with methylprednisolone (MP) and its liver-targeted macromolecular prodrug (13,14), dextran-MP (DMP), significantly attenuates the preservation-induced KC damage. Because, corticosteroids are also shown (15) to have a protective effect on hepatocytes, the second goal of the study was to investigate the effects of donor pretreatment with MP or DMP on the kinetics of ICG in cold-preserved IPRLs. Our hypothesis was that an increase in the duration of preservation would result in a progressive decrease in the hepatic uptake and/or biliary excretion of ICG, which would be attenuated by MP and/or DMP pretreatment.

MATERIALS AND METHODS

Chemicals

Dextran (MW, 73 kDa) with polydispersity of <2, ICG, sodium taurocholate, and kits for measurement of alanine aminotransferase (ALT), aspartate aminotransferase (AST), acid phosphatase (ACP), and lactate dehydrogenase (LDH) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). 6α-Methylprednisolone (MP) and MP succinate (MPS) were purchased from Steraloids (Wilton, NH, USA). Belzer's University of Wisconsin (UW) solution (Viaspan)

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was obtained from Dupont Pharma (Wilmington, DE, USA). All other reagents were analytical grade and obtained through commercial sources.

Dextran–methylprednisolone succinate (DMP), containing 8% (w/w) MP, was synthesized, purified, and characterized as described before (16). The dosing solutions (5 mg/ml, MP equivalent) of MP and DMP were prepared in a mixture of PEG400:water:ethanol (6.0:2.8:1.2, v/v) and distilled water, respectively.

Experimental Protocol

All procedures involving animals used in this study were consistent with the guidelines set by the National Institute of Health (NIH Publication No. 85-23, revised 1985) and approved by our Institutional Animal Care and Use Committee.

A total of 44 male Sprague-Dawley rats (body weight, 232–284 g) were used in this study that consisted of 11 groups (n = 4/group). Nine groups of animals received saline (control) or single 5-mg/kg doses (MP equivalent) of MP or DMP via tail vein 2 h prior to liver isolation (3 groups/treatment). This dose was selected because in a previous study (12) we showed that a similar dose would result in a substantial attenuation of KC activation due to cold I/R. For each treatment, the three groups of isolated livers were then preserved in cold for 24, 48, or 72 h before the ICG kinetic study. A tenth group of animals (unpreserved control, n = 4) received saline injection, and livers were used for ICG study without any preservation.

We used saline injections in all of our control groups (0, 24, 48, or 72 h of preservation) to account for the possible effects of injection procedure on the study outcomes and to avoid unnecessary duplication of control groups by using both MP and DMP vehicles in separate groups of animals for all the preservation times. This protocol, however, assumes no differences between the effects of vehicles. Because the MP vehicle contained PEG400 (60%, v/v) and ethanol (12%, v/v) in addition to water, its effect on the ICG kinetics was also studied in an additional unpreserved liver group and compared with those after the saline injection. As demonstrated later in the "Results" section, the kinetics of ICG was not affected by the vehicle used, supporting our assumption.

Liver Harvest, Preservation, and Reperfusion

These were similar to the procedures reported by us recently (12). Briefly, rats were anesthetized (ketamine:xylazine), and the liver perfused via the portal vein with 75 ml of cold (4°C) UW solution (5 ml/min). The livers were excised and stored in 50 ml of UW solution at 4°C to simulate cold preservation.

Following 0 (no preservation), 24, 48, or 72 h of cold-preservation, the livers were washed with 20 ml of lactated Ringer's solution to remove the UW solution. The initial 1-ml samples of the effluent produced during this wash were collected and stored either at 4°C for the analysis of ALT and LDH or at –80°C for the analysis of AST and ACP. The livers were then mounted onto a water-jacketed, all-glass perfusion system (Radnoti Glass Technology Inc., Monrovia, CA, USA) kept at 37°C. The perfusate was Krebs-Henseleit bicarbonate buffer (pH 7.4) containing 1.2 g/L glucose and 75 mg/ml of sodium taurocholate, which was oxygenated with a 95:5 oxygen:carbon dioxide mixture. The perfusion was performed in a single-pass manner with a flow rate (Q) of 30 ml/min (~3–4 ml min^{-1} g^{-1} of liver weight). Following mounting, the livers were allowed to stabilize for ~15 min before start of the experiments which continued for an additional 90 min.

ICG Infusion and Sample Collection

A concentrated solution of ICG (400 μg/ml in 2% ethanolic saline) was prepared fresh before the start of each perfusion. The solution was then infused at a constant rate of 0.3 ml/min (120 μg/min) into the portal vein catheter, resulting in inlet (portal vein) concentrations of ~4 μg/ml. Subsequently, inlet (~1 ml) and outlet (~3 ml) samples were collected at 5-min intervals for the first 30 min, at which time ICG infusion was stopped. The infusion of 30 min was selected based on previous studies reporting that the ICG steady-state concentrations in the outlet of single-pass livers are achieved in <25 min (17,18). Furthermore, our preliminary studies using preserved livers supported our selection of infusion time. The perfusion of the liver was then continued for an additional 60 min in the absence of ICG, and outlet samples were collected at 35, 40, 45, 50, 60, 70, and 90 min after the start of perfusion. Immediately after sample collection, a portion (~0.5 ml) of the inlet or outlet samples were diluted with an equal volume of methanol and stored at –80°C for the analysis of ICG. The remaining sample was used for the analysis of ALT, AST, LDH, or ACP contents.

In addition to the inlet and outlet samples, bile samples were also collected in pre-weighed microcentrifuge tubes at 30-min intervals. The samples were then diluted (3–5-fold) with a mixture of perfusate:methanol (1:1) and stored at –80°C for subsequent analysis of ICG. Inlet pressure was monitored continuously with a pressure transducer and recorded at each sampling point. Following perfusion for 90 min, the liver was blotted dry, weighed, and stored at –80°C for measurement of DMP and/or free MP concentrations.

Sample Analysis

Indocyanine green is reportedly (19) unstable in aqueous solutions. In a preliminary study, we noticed that while ICG was unstable in the perfusate, addition of an equal volume of methanol immediately after sample collection rendered it completely stable (~100% recovery) for at least 8 days when samples were stored at –80°C. Perfusate and biliary ICG concentrations were, therefore, measured within a week of collection using a previously reported (20) reversed-phase high performance liquid chromatography (HPLC) system with minor modifications. Briefly, 50 μl of the methanol-diluted (1:1) samples were directly injected into an HPLC system consisting of a Microsorb-MV C-18 column (Varian Inc., Walnut Creek, CA, USA) with a mobile phase of 0.05 M phosphate buffer (pH 5.3): acetonitrile (50:50, v/v). The assay had a lower limit of quantitation of 0.1 μg/ml of perfusate and a CV and error of <5% (n = 5).

The quantitation of hepatic concentrations of DMP and free MP was performed using size-exclusion (21) and reversed-phase (22) HPLC methods, respectively, modified for hepatic tissue matrix (13). The perfusate enzyme levels were determined using commercially-available spectrophotometric kits.

Pharmacokinetic Analysis

The experimental values of inlet concentrations of ICG for all the samples collected during the first 30 min were used to calculate the average inlet concentration of the marker (C_{in}) for each liver. Similarly, the average outlet concentration (C_{out}) was calculated from the last three outlet samples obtained during the ICG infusion (20, 25, and 30 min). The area under the outlet perfusate concentration-time curve ($AUC_{out}^{0-\infty}$) was estimated by trapezoidal rule from time zero to the last measurable concentration (C_{last}), with extrapolation to time infinity using the apparent ICG efflux rate constant (K). The latter was estimated from the log linear portion of the perfusate concentration-time course of ICG during the washout period. The hepatic extraction ratio (E) of ICG was estimated in two different ways. The first method (E_{ss}) was based on C_{in} and C_{out} values at steady state:

$$E_{ss} = \frac{C_{in} - C_{out}}{C_{in}} \tag{1}$$

In the second method, however, the total input and output of ICG during the entire perfusion (ICG infusion and washout) was used to calculate an average E (E_{av}) based on the following equation:

$$E_{av} = \frac{D_{in}^{0-30} - D_{out}^{0-\infty}}{D_{in}^{0-30}} \tag{2}$$

where D_{in}^{0-30} and $D_{out}^{0-\infty}$ refer to the total ICG dose infused during the infusion time (T_{inf}) of 30 min ($D_{in}^{0-30} = C_{in} \times Q \times T_{inf}$) and total amount of ICG recovered in the perfusate from zero to infinity ($D_{out}^{0-\infty} = AUC_{out}^{0-\infty} \times Q$), respectively. The amount of ICG effluxed from the liver into the perfusate after the cessation of ICG infusion ($D_{out}^{30-\infty}$) was estimated from the equation $D_{out}^{30-\infty} = AUC_{out}^{30-\infty} \times Q$, where $AUC_{out}^{30-\infty}$ is the AUC of ICG in the outlet perfusate from 30 min to infinity. The hepatic (CL_h) and intrinsic (CL_{int}) clearance values of ICG were calculated according to the following equations, based on a well-stirred hepatic model (23) and E_{av} values:

$$Cl_h = Q \cdot E_{av} \tag{3}$$

$$Cl_{int} = \frac{Cl_h}{1 - E_{av}} \tag{4}$$

The concentration of ICG in the liver tissue at any time during or after the cessation of ICG infusion (C_{liver}^{t}) was estimated indirectly from the following equation:

$$C_{liver}^{t} = \frac{D_{in}^{0-T} - D_{out}^{0-t}}{V_{liver}} \tag{5}$$

where D_{in}^{0-T}, D_{out}^{0-t}, and V_{liver} represent the amount of ICG infused up to time T, the amount recovered in the perfusate up to the sampling time t, and the experimental volume of the liver, respectively. The time T is equal to t during the infusion of ICG and equal to 30 when $t \geq 30$ min. This estimation assumes that the biliary excretion of ICG during the short perfusion time of 90 min is minor relative to the amount of ICG effluxed back to the perfusate.

The AUCs were also estimated for ACP, ALT, and AST concentrations in the outlet perfusate and pressure in the inlet perfusate by linear trapezoidal rule for the duration of perfusion (90 min). The average portal resistance during the 0–90 min perfusion period was estimated for each liver from the following equation:

$$\text{Portal Resistance (mmHg} \cdot \text{min} \cdot \text{g/ml)} = \frac{\text{Average Portal Pressure (mmHg)}}{\text{Perfusate Flow Rate (ml min}^{-1}\text{ g}^{-1}\text{)}} \tag{6}$$

where the average portal pressure was first estimated by dividing the pressure AUC by the perfusion time (90 min).

Statistical Analysis

The effects of duration of preservation (0, 24, 48, or 72 h) or drug treatments (control, MP, or DMP) on the ICG kinetics and liver viability markers were assessed using ANOVA. In the presence of a significant result, pairwise comparison of means was performed using Fisher's post-hoc test. A paired, two-tailed t test was used for comparison of E_{ss} and E_{av} values obtained through two different methods. Finally, the effect of vehicle was tested by comparing kinetic parameters of ICG after the saline or MP vehicle injection using an unpaired, two-tailed t test. All tests were performed at a significance level (α) of 0.05. Data are presented as mean ± SE.

RESULTS

Effects of Duration of Ischemia on Viability Markers and ICG Kinetics

The concentrations of hepatic enzymes along with portal resistance values in livers preserved for 0–72 h are presented in Table I. Cold ischemia resulted in an increase in the concentrations of all three enzymatic markers measured in the solution exiting the liver during the initial flush immediately after the preservation. The most dramatic increase (>60-fold) was, however, observed with ACP, whereas ALT and AST concentrations were maximally increased only by ~3 fold (Table I). Additionally, the ACP concentrations in the effluent of livers preserved for 48 and 72 h were significantly higher than those in livers preserved for 24 h (Table I). The data obtained during the reperfusion of the livers were qualitatively similar to those obtained in the initial flush (Table I). However, preservation caused similar increases (5–7-fold) in all four enzymatic markers measured during the reperfusion (ACP, ALT, AST, and LDH). Furthermore, not only were the concentrations of all four enzymes in the 72-h preserved livers different ($p < 0.05$) from those in the unpreserved livers, but also they differed significantly ($p < 0.05$) from those preserved for 24 and 48 h (Table I). As for portal resistance, no significant differences were observed between different preservation groups (Table I).

The time courses of ICG concentration in the inlet and outlet perfusate and liver tissue of IPRLs are shown in Fig. 1. The inlet concentrations remained relatively constant in each liver during the 30 min of infusion and were between 3 to 4 μg/ml in different preservation groups (Fig. 1). Except for the unpreserved group, ICG concentrations in the outlet perfusate started to rise within 5 min and reached relatively constant levels at ~15 min. However, the rise in the outlet concentra-

Table I. Cellular and Hemodynamic Marker Levels in Initial Flush Solution and in the Perfusate of Rat Livers Subjected to Cold Preservation for 0, 24, 48, or 72 h (n = 4/Group)[a,b]

Marker	Duration of cold-preservation (h)			
	0	24	48	72
		Initial flush data		
ACP Conc.[c]	1.1 ± 0.4	28 ± 7[g]	67 ± 6[g,h]	72 ± 5[g,h]
ALT Conc.[c]	18 ± 1	46 ± 0[g]	45 ± 7[g]	61 ± 10[g]
AST Conc.[c]	24 ± 3	26 ± 14	78 ± 19[g,h]	65 ± 13
		Perfusate data		
ACP AUC[d]	40 ± 5	68 ± 22	120 ± 15[g,h]	220 ± 9[g,h,i]
ALT AUC[d]	420 ± 160	1600 ± 160[g]	1200 ± 200	2800 ± 640[g,h,i]
AST AUC[d]	480 ± 62	1200 ± 300	1200 ± 180	2300 ± 500[g,h,i]
LDH Conc.[c,e]	0.15 ± 3.4	51 ± 20	125 ± 44[g]	224 ± 40[g,h,i]
Portal resistance[f]	2.6 ± 0.6	4.4 ± 0.3	4.4 ± 1.3	4.3 ± 0.79

[a] ACP, acid phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; LDH, lactate dehydrogenase.
[b] Values are mean ± SE.
[c] IU/ml.
[d] IU · min/ml.
[e] LDH concentration was measured only at the end of perfusion (90 min).
[f] mmHg · min · g/ml
[g] Significantly different ($p < 0.05$) from the unpreserved (0 h) group.
[h] Significantly different ($p < 0.05$) from the 24-h group.
[i] Significantly different ($p < 0.05$) from the 48-h group.

tions of ICG in unpreserved group was slower than that in the cold-preserved livers, resulting in a lack of detection of the marker at 5 min in three out of the four unpreserved livers and an apparent delay in reaching steady state (Fig. 1). Nevertheless, C_{out} values, which were estimated by the average outlet concentrations at the last three sampling points during ICG infusion (20, 25, and 30 min), were not significantly different among the four studied preservation times ($p > 0.05$; ANOVA). After cessation of ICG infusion, the outlet concentrations of the marker declined relatively slowly, indicating that ICG is returning from the liver tissue back to the perfusate (Fig. 1). Additionally, an increase in the duration of

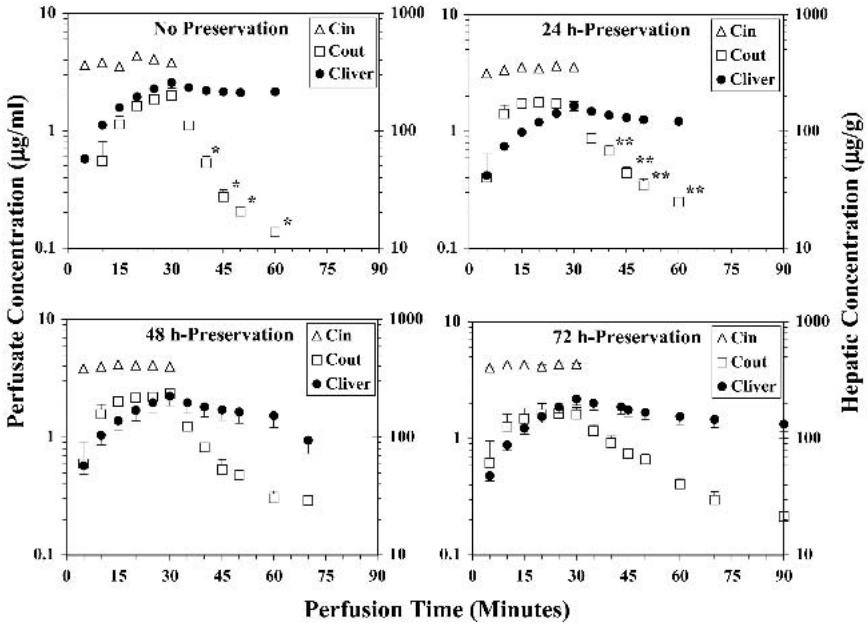

Fig. 1. The time courses of inlet (C_{in}) and outlet (C_{out}) perfusate and liver tissue (C_{liver}) concentrations of ICG in isolated livers subjected to 0 (top left), 24 (top right), 48 (bottom left), or 72 (bottom right) h of cold ischemia followed by 90 min of reperfusion (n = 4/group). Symbols and bars represent the average and standard error values, respectively. *Statistically different ($p < 0.05$) from the corresponding concentrations in 24-, 48-, and 72-h preserved livers; **statistically different ($p < 0.05$) from the corresponding concentrations in 72-h preserved livers.

Table II. Kinetic Parameters of ICG in Isolated Perfused Rat Livers Subjected to Cold Preservation for 0, 24, 48, or 72 h (n = 4/Group)[a,b]

Preservation time (h)	E_{ss}	E_{av}	CL_h ml min^{-1}kg^{-1}	CL_{int} ml min^{-1}kg^{-1}	$D_{out}^{30-\infty}$ %dose	K min^{-1}
0	0.53 ± 0.03	0.57 ± 0.01	63 ± 2	150 ± 6	16 ± 1	0.071 ± 0.014
24	0.50 ± 0.04	0.38 ± 0.05[c,d]	43 ± 7	73 ± 16[c]	24 ± 3	0.041 ± 0.008[c]
48	0.45 ± 0.01	0.32 ± 0.07[c]	39 ± 9[c]	62 ± 18[c]	28 ± 4[c]	0.039 ± 0.009[c]
72	0.62 ± 0.07	0.36 ± 0.07[c,d]	40 ± 7[c]	66 ± 16[c]	35 ± 2[c]	0.020 ± 0.002[c]

[a] E_{ss}, extraction ratio obtained by Eq. 1; E_{av}, extraction ratio obtained by Eq. 2; CL_h, hepatic clearance; CL_{int}, intrinsic hepatic clearance; $D_{out}^{30-\infty}$, amount of ICG recovered during the washout period; K, apparent rate constant for the efflux of ICG from the liver during the terminal phase of the washout period.

[b] Values are mean ± SE.

[c] Significantly different from unpreserved livers (preservation time of zero): $p < 0.05$, ANOVA, followed by Fisher's test.

[d] Significantly different from E_{ss}: $p < 0.05$, paired, two-tailed t test.

preservation caused a progressively higher and more sustained level of ICG in the outlet perfusates during the washout period (Fig. 1). Also included in Fig. 1 are the estimated concentrations of ICG in the liver tissue, which were severalfold (≥100-fold at ≥30 min) higher than the concentrations of the marker in the outlet perfusate. The liver concentrations of ICG progressively increased with time during the infusion of ICG and declined slowly thereafter (Fig. 1).

The kinetic parameters of ICG in livers preserved for 0–72 h are presented in Table II. When E was estimated from the C_{in} and C_{out} values (E_{ss}), it ranged from 0.45 to 0.62 and was not significantly affected by the duration of preservation. However, cold preservation caused a modest (30–40%) decrease in E_{av}, which was estimated from the total input and output data (Table II). Although the E_{ss} and E_{av} values were similar for the unpreserved livers, the E_{av} values were consistently lower than the corresponding E_{ss} values for the preserved livers (Table II). Cold preservation also resulted in a decrease in the CL_h and CL_{int} values, which were estimated from E_{av}. The amount of ICG recovered during the washout period ($D_{out}^{30-\infty}$) progressively increased from 16 to 35% of the administered dose when the preservation time was increased from 0 to 72 h ($p < 0.05$). Similarly, the apparent efflux rate constant (K) significantly declined ($p < 0.05$) in the cold-preserved livers, resulting in harmonic half lives of 9.8, 17, 18, and 35 min in the livers preserved for 0, 24, 48, and 72 h, respectively (Table II).

The bile data are presented in Fig. 2. The flow rates in the unpreserved livers (0 h) substantially decreased during the second and third 30-min collection intervals, compared with the values during the first 30-min period (Fig. 2, top). As for the effect of cold ischemia, a substantial decline in the bile flow rates at all the collection intervals and for all the preservation times was observed in comparison to the flow rates in the unpreserved livers (0 h) (Fig. 2, top). In contrast to the bile flow rates (Fig. 2, top), the amount of ICG excreted into the bile of unpreserved livers increased with an increase in the collection interval (Fig. 2), indicating a delay in the appearance of ICG in bile. Additionally, cold preservation caused drastic declines ($p < 0.05$) in the amount of ICG excreted in bile, with no detectable ICG in the livers preserved for 72 h (Fig. 2, bottom).

Effects of Donor Pretreatment on Viability Markers and ICG Kinetics

Pretreatment of rat donors with MP or DMP did not significantly ($p > 0.05$) affect the levels of ALT, AST, and LDH and portal resistance in either the initial flush or the perfusate (data not shown); the only exception was a 79% decline ($p < 0.05$) in the initial flush levels of AST by MP pretreatment in the 72-h preserved livers. In contrast, both MP and DMP resulted in significant declines in the concentration of ACP, a marker of KC activation (24), in both the initial flush and the perfusate (Fig. 3). For the initial flush, the maximum degree of reduction in the ACP levels (~90%) was observed at 24 h for both MP- and DMP-treated groups ($p < 0.05$) (Fig. 3, top), although the effects of pretreatments were significant at all the preservation times. For the AUC of ACP

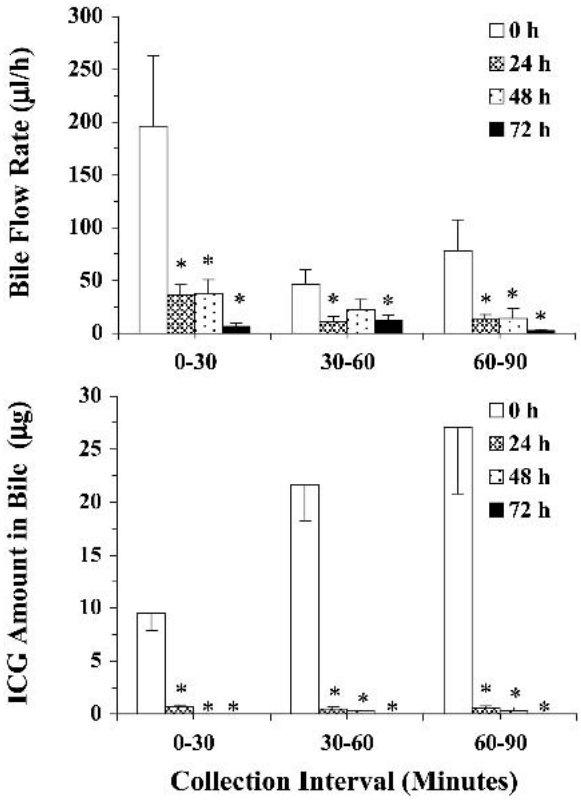

Fig. 2. Bile flow rates (top) and ICG amounts excreted into bile (bottom) at different collection intervals in isolated livers subjected to 0, 24, 48, or 72 h of cold ischemia followed by 90 min of reperfusion (n = 4/group). Columns and bars represent the average and standard error values, respectively. Statistical comparisons are based on ANOVA followed by Fisher's post hoc analysis. *Significant differences between the cold-preserved livers and unpreserved controls.

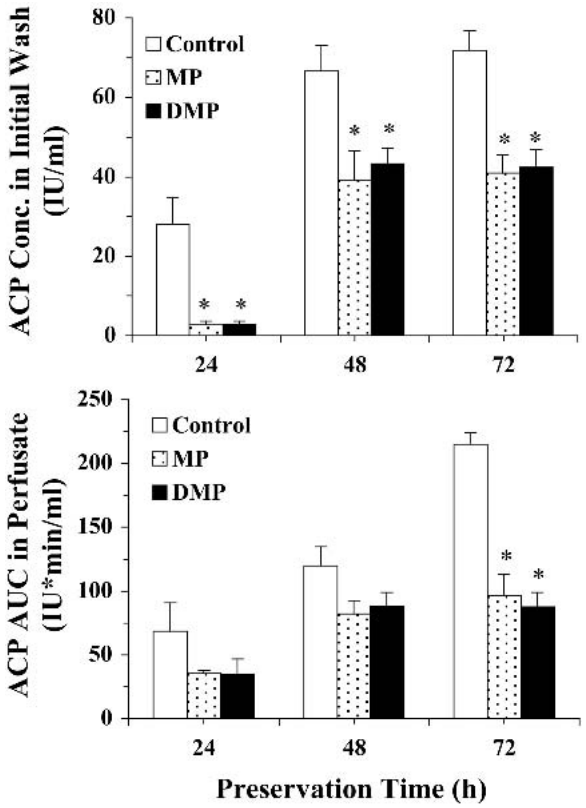

Fig. 3. Acid phosphatase (ACP) concentrations in the initial wash solution (top) and perfusate AUC (bottom) in livers isolated from pretreated rats (Control, MP, DMP) and subjected to 24, 48, or 72 h of cold ischemia followed by 90 min of reperfusion. The rats were pretreated intravenously, 2 h prior to liver harvest, with a single 5 mg/kg dose (MP equivalent) of MP or DMP or with saline (Control) (n = 4/group). Columns and bars represent the average and standard error values, respectively. Statistical comparisons are based on ANOVA followed by Fisher's post hoc analysis. *Significant differences between the treated groups and respective controls.

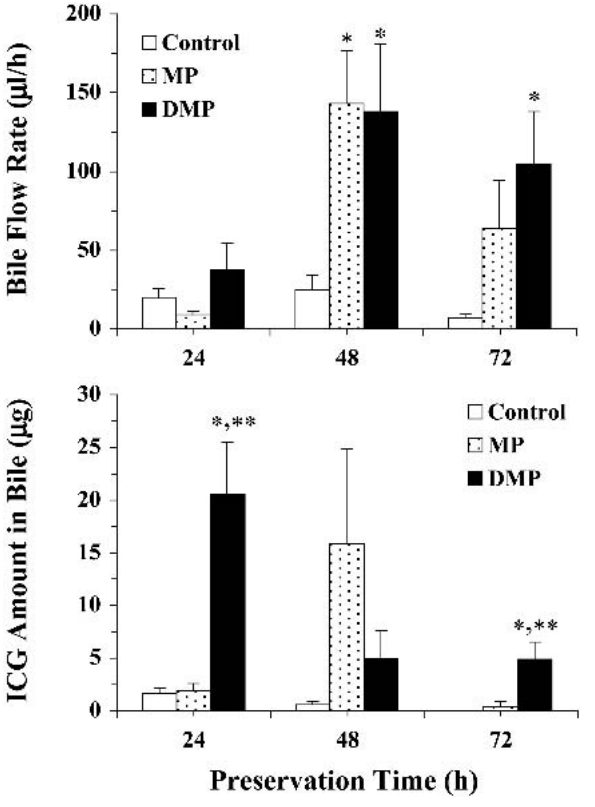

Fig. 4. The time-averaged (0–90) bile flow rates (top) and total ICG amounts excreted into bile from zero to 90 min (bottom) in livers isolated from pretreated rats (Control, MP, DMP) and subjected to 24, 48, or 72 h of cold ischemia followed by 90 min of reperfusion. The rats were pretreated intravenously, 2 h prior to liver harvest, with a single 5 mg/kg dose (MP equivalent) of MP or DMP or with saline (Control) (n = 4/group). Columns and bars represent the average and standard error values, respectively. Control values are the time (0–90)-averaged values presented in Fig. 2 for each interval. Statistical comparisons are based on ANOVA followed by Fisher's post hoc analysis. *Significant differences between the treated groups and respective controls; **significant differences between the DMP and MP treated groups.

in the perfusate, pretreatment-induced declines were observed for all the preservation times, with only the decline in the 72-h group (~60% for both MP and DMP) reaching statistical significance (Fig. 3, bottom).

As for the kinetics of ICG, the values derived from the perfusate data listed in Table II were not significantly affected by pretreatment with either MP or DMP (data not shown). However, significant effects of pretreatments were evident for the bile data (Fig. 4). For clarity, bile data for different pretreatment and preservation time groups are presented as a single point for the entire perfusion (0–90 min) (Fig. 4). However, the pattern of bile flow rate and ICG excretion for individual collection intervals were similar to those presented for controls in Fig. 2. Although DMP did not significantly affect the bile flow rates in the 24-h preserved livers, it resulted in substantial ($p < 0.05$) increases of 6- and 15-fold in the bile flow rates of 48- and 72-h preserved livers, respectively (Fig. 4, top). For MP pretreatment, the data showed a statistically significant ($p < 0.05$) 6-fold increase in the bile flow rates only at the 48 h preservation time (Fig. 4, top). In addition to the pretreatment-induced changes in the bile flow rates, pretreatment of rats with DMP caused significant ($p < 0.05$) increases (>12-fold) in the biliary excretion of ICG after 24 and 72 h of cold preservation (Fig. 4, bottom). In contrast, biliary excretion of ICG was not significantly altered by MP pretreatment (Fig. 4, bottom).

The hepatic concentrations of the prodrug DMP and the regenerated parent drug MP at the end of perfusion are depicted in Fig. 5 for the rats pretreated with the prodrug. The concentrations of DMP were relatively high (~30 µg/g) for all the studied preservation times. In addition to DMP, regenerated MP was detected in the liver of all the groups, although its concentrations (<0.5 µg/g) were much lower than those of the prodrug (Fig. 5). Furthermore, the concentrations of the regenerated MP declined with an increase in the preservation time (Fig. 5). Following administration of equivalent doses of the parent drug MP, the hepatic concentrations of MP were below the limit of quantitation of the assay (<0.1 µg/g) in all the preservation groups.

Effects of MP Vehicle on ICG Kinetics

Because the MP vehicle contained PEG400 and ethanol, in addition to water, its effects on the hepatic disposition of

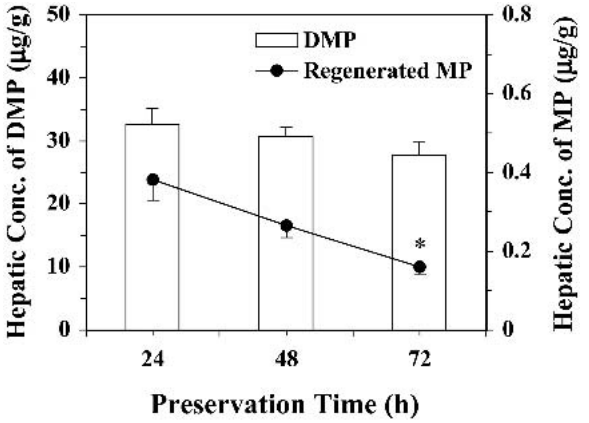

Fig. 5. The concentrations of the prodrug (DMP, columns) and regenerated drug (MP, circles) in the liver tissues collected from donors pretreated with 5 mg/kg DMP (MP equivalent) and subjected to 24, 48, or 72 h of cold ischemia followed by 90 min of reperfusion. Columns and circles represent the average values and bars represent standard error values. Statistical comparisons are based on ANOVA followed by Fisher's post hoc analysis. *Significant difference between the 72- and 24-h groups.

ICG were studied. The kinetic parameters of ICG in the livers of rats injected with MP vehicle were E_{ss}, 0.54 ± 0.07; E_{av}, 0.56 ± 0.06; CL_h, 65 ± 3 ml min^{-1} kg^{-1}; CL_{int}, 150 ± 16 ml min^{-1} kg^{-1}; $D_{out}^{30-\infty}$, 14% ± 2%; and K, 0.10 ± 0.02 min^{-1}. All of these values were similar to those after the injection of saline (Table II) ($p > 0.05$). Additionally, the total amounts of ICG excreted in bile during the perfusion (0–90 min) were the same ($p > 0.05$) after the MP vehicle (54 ± 10 μg) or saline (58 ± 8 μg) injections.

DISCUSSION

Liver injury due to cold I/R compromises the functional status of hepatocytes (3), which play a crucial role in the disposition and metabolism of many drugs and endogenous compounds. Cold preservation depresses high-energy nucleotide content and concomitantly augments protease activity (3) and oxidant stress (25) in hepatocytes. Given that sinusoidal transport, metabolism, and biliary excretion of many drugs are energy-dependent processes, changes in hepatic disposition of drugs may be expected following cold I/R. Indeed, our results using ICG show that both the hepatic extraction and biliary excretion of the marker are altered after cold I/R (Table II, Figs. 1 and 2).

The hepatic extraction of ICG and most other drugs in IPRL is normally estimated from C_{in} and C_{out} values using Eq. 1 (10,17,23). Although this method considers steady-state concentration data, it is not sensitive to the rate at which the steady state is achieved. Consequently, in the absence of significant changes in C_{in} and C_{out} values, our estimated E_{ss} values (Table II) were not sensitive to the apparent preservation-induced changes in the outlet profiles of ICG (Fig. 1). This is consistent with a recent preliminary report (26) on the lack of effect of cold preservation on the hepatic extraction of ICG in dog livers. In contrast, when we estimated the average extraction ratio during the entire perfusion period (E_{av}), preservation resulted in a modest decrease in the hepatic extraction of ICG (Table II). It should be noted that our estimation of E_{ss} value for the unpreserved livers (Table II) might have been slightly overestimated because of the delay in the rate of achievement of steady state for this group, as opposed to cold-preserved livers (Fig. 1). However, this overestimation, if any, cannot explain the lack of reduction in E_{ss} due to cold preservation (Table II).

The differences between the E_{ss} and E_{av} values are most likely due to the inclusion of washout data in the latter. Available reports (10,17) on the ICG disposition in single-pass IPRLs have all used the data during the infusion of the marker; we are not aware of any report on the ICG kinetics during a washout period. Because ICG is not metabolized, excretion into bile and/or sinusoidal efflux is the only likely mechanism for its removal from the hepatocytes. Therefore, in addition to biliary excretion, we studied the effects of preservation injury on the efflux of the marker during a washout period. A substantial fraction of the ICG dose (16–35%) was extruded from the liver into the perfusate during the washout period (Table II). The significant increases in the efflux amount of ICG as a result of 24–72 h preservation (Table II) suggests that the preservation-induced decreases in E_{av} values (Table II) are mostly due to the increases in the efflux of the marker during the washout period.

One may argue that an I/R-mediated increase in the efflux of ICG should be present during both the ICG infusion and washout period, thus affecting both E_{ss} and E_{av} values similarly. The lack of differences between the E_{ss} values of preserved and unpreserved livers (Table II), therefore, may suggest that the I/R-mediated increases in the efflux of ICG are more prominent at later times during the reperfusion of the liver. Indeed, a recirculating IPRL study (27) using a bolus dose of bromosulfophthalein, a marker similar to ICG, is in agreement with this postulate; after an initial rapid decline, the perfusate concentrations of the marker started to rise again at ~50 min of reperfusion in livers subjected to 30–60 min of warm ischemia, resulting in a secondary peak. Because reperfusion itself induces significant damage to the ischemic livers, it is plausible that with an increase in reperfusion time in our experiments, the damage to the ischemic liver increases, causing higher extrusion of ICG at later time points. Therefore, the differences between E_{ss} and E_{av} in their sensitivity to cold preservation are most likely due to preservation-induced changes in the efflux of ICG at later times during the perfusion, an event that cannot be reflected in E_{ss} values.

The mechanism(s) for the I/R-induced increases in the efflux of ICG (Table II) is not clear from our data. However, alterations in the cellular transporters for ICG (e.g., Oatp1) and/or damage to the integrity and function of hepatocyte tight junctions (27) may be responsible. Nevertheless, regardless of the mechanism, alterations in the net ICG uptake following cold I/R portend likelihood for disturbances in the disposition of many drugs following transplantation of livers subjected to prolonged preservation.

Bile flow rate has been shown (11) to discriminate between viable and non-viable livers in transplant models. Additionally, ICG is used as a marker of hepatocellular function, and its biliary excretion is reported (26) as an index of ischemic injury during the early reperfusion period after liver transplantation. Therefore, the drastic declines in bile flow and biliary ICG excretion with an increase in the duration of preservation (Fig. 2) indicate severe impairment of canalicular transport function.

Although E_{av} and $D_{out}^{30-\infty}$ values responded modestly to cold I/R injury (Table II), biliary excretion of ICG was by far the most sensitive parameter for this injury (Fig. 2). The cytoplasmic transport and canalicular excretion of ICG are mediated by microtubules (28) and multidrug resistance P-glycoprotein 2 (Mdr2) (9), respectively. Cold I/R causes a decrease in ATP content (3) and enhances activation of many proteases (3,15) in hepatocytes. Because microtubule stability and Mdr2 function are dependent on ATP (28), a decrease in high-energy nucleotides following I/R may attenuate these pathways, hence decreasing ICG excretion into bile. Moreover, degradation of microtubules by various proteases and Ca^{2+} dependent pathways (28), enhanced by I/R, may contribute to the decreased ICG transport. These alterations can also have a deleterious effect on post-ischemic bile formation given that microtubules and Mdr2 play an important role in maintaining bile flow (28) and composition (9), respectively.

The rapid decline in the bile flow rates of unpreserved livers during the 90-min perfusion (Fig. 2, top) has not been observed in our previous studies in the absence of ICG (12,14), suggesting that ICG at the levels used in our study reduces, at least in part, the bile flow rate. Indeed, other studies (29) have shown that ICG decreases bile flow *in vivo*. Furthermore, the increase in the biliary excretion of ICG at later intervals when the infusion of ICG was stopped (Fig. 2, bottom) indicates that the biliary excretion of ICG is delayed. Therefore, during the relatively short period of perfusion (90 min), only a small amount of the marker was recovered in bile (Fig. 2, bottom), as opposed to more substantial biliary excretion of the marker under *in vivo* conditions (30) or in IPRLs infused with lower concentrations of ICG (10).

Because corticosteroids are shown (15) to be protective of hepatocyte functions during warm ischemia, we examined whether the deleterious effects of cold I/R on hepatocytes could be ameliorated by pretreatment of liver donors with MP or its liver-targeted (13,14) macromolecular prodrug, DMP. The significant improvements in bile flow rate and ICG biliary excretion in the liver of rats pretreated with the prodrug (Fig. 4) was associated with relatively high concentrations of DMP and regenerated MP in these livers (Fig. 5). Therefore, the less prominent effects of the parent drug injection on restoring the bile flow rate and biliary excretion of ICG (Fig. 4) may be attributed to its low levels (below detection) in the liver of MP-pretreated rats.

In addition to improvements in hepatocyte viability, cold I/R-mediated activation of KC, as measured by ACP levels (24), was attenuated by pretreatment with MP and DMP (Fig. 3). These results are in agreement with our recent study (12), wherein the levels of TNF-α, a cytokine released following KC activation, were significantly reduced after pretreatment with MP or DMP. However, the attenuating effects of DMP were more prominent than those of MP (12). Because sinusoidal endothelial cells are also crucially involved in the pathogenesis of I/R injury (4), and there is a high degree of communication among liver cells (31), the corticosteroid pretreatment may also have a protective effect on these cells. Studies using specific markers of endothelial cell function [e.g., hyaluronic acid (32)], are currently underway to test this hypothesis.

Our previous study on KCs (12) and the current study on hepatocytes suggest that pretreatment of donors with the liver-targeted prodrug of MP (DMP) may be an effective approach in reducing the I/R-mediated injury to the livers subjected to prolonged preservation. Furthermore, the relatively high hepatic concentrations of the prodrug and regenerated MP following prodrug pretreatment (Fig. 5) may also contribute to the desired local immunosuppression after the liver is transplanted. This postulate will be tested in future studies using experimental models of liver transplantation.

In conclusion, cold hepatic I/R injury increased the efflux and decreased the extraction ratio and biliary excretion of ICG in an IPRL model. Among the estimated kinetic parameters, the excretion of ICG into bile was the most sensitive marker of liver damage. Pretreatment of liver donors with a liver-targeted prodrug of methylprednisolone (DMP) significantly attenuated cold I/R-mediated decrease in the biliary excretion of ICG. Further investigations are needed to determine the effectiveness of donor pretreatment with corticosteroids in experimental models of liver transplantation.

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