RETENTION OF TRANSPORTER ACTIVITIES IN CRYOPRESERVED, ISOLATED RAT HEPATOCYTES

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(Received September 3, 2002; accepted January 2, 2003)

This article is available online at http://dmd.aspetjournals.org

ABSTRACT:
The success of cryopreservation of isolated hepatocytes with existing methodologies is assessed with respect to the retentivity of cell integrity/viability (defined by trypan blue) and metabolic activities upon thawing in comparison to those of freshly prepared cells. But the ability of the cryopreserved cells to transport xenobiotics relative to that of freshly prepared cells has not been investigated. In this study, we optimized our previous methodology for cryopreservation and evaluated the metabolism and transport of thawed hepatocytes. Half of the freshly, isolated rat hepatocytes prepared by collagenase perfusion were immediately used for preservation and evaluated the metabolism and transport of hepatocytes cryopreserved by a programmed-freezing protocol using an optimized, programmed-freezing protocol, which was developed to minimize the prolonged release of latent heat during freezing. With the exception of the UGT probe, no significant difference (P > 0.05) was found in both metabolism and transport with freshly isolated versus cryopreserved hepatocytes upon thawing. In conclusion, we have demonstrated for the first time that thawed rat hepatocytes cryopreserved by a programmed-freezing protocol retain drug transport activities.

Drug transport across the hepatocyte plasma membranes is a key factor in hepatic clearance. Hepatobiliary transport of endogenous and exogenous compounds is mediated by the co-ordinated action of multiple transport systems present at the sinusoidal (basolateral) and canalicular (apical) membrane domains of hepatocytes. The lipophilic properties of drugs enable them to cross the sinusoidal membrane by passive diffusion. However, for others, entry into the hepatocyte can be facilitated via a variety of sinusoidal transporters that augment passive diffusion. Such drugs with molecular weights >400 tend to be hydrophilic since they usually contain polar groups and are ionized (either anionic or cationic) (Meijer et al., 1990; Aytton and Morgan, 2001). These drugs enter the liver by sinusoidal drug transporters, including the sodium-dependent taurocholate cotransporting polypeptide (NTCP)2 (Hagenbuch and Meier, 1994), members of the organic anion-transporting polypeptide (OATP) (Bossuyt et al., 1996; Cattori et al., 2001), and the organic cationic transporter (Grünemann et al., 1994). Biliary elimination of drugs is mediated by different ATP-binding cassette-transporters as exemplified by the multidrug-resistance P-glycoprotein (MDR1, MDR2) for neutral and cationic compounds (Meijer et al., 1997), and the canalicular multi-drug resistance-associated transporter (MRP2) (Payen et al., 2000) for anionic and conjugated drugs.

In our laboratory, rat and human hepatocytes have been routinely used for studies related to hepatic metabolism and transport of xenobiotics. For transport studies, freshly isolated hepatocytes have been used to investigate drug uptake to determine the role of transporters in the overall drug disposition by the liver (Tan et al., 1999; Abu-Zahtah et al., 2000; Kusuhara and Sugiyama, 2002; Meng et al., 2002). To this effect, freshly isolated rat hepatocytes have been commonly used since several reports have demonstrated good correlation between results obtained with rat hepatocytes with those from perfused liver and whole animal studies (Tan et al., 1999; Abu-Zahtah et al., 2000; Kusuhara and Sugiyama, 2002). One of the limitations of this technique is the requirement for isolation of cells from fresh tissue every time an experiment is to be conducted. Another major hurdle is the scarcity of liver tissue available for some species, especially human. It would be highly desirable if daily preparation could be avoided. This may be achieved by cryopreservation of surplus hepatocytes after isolation so that they can be stored for use when needed. We and others have already demonstrated that cryopreserved hepatocytes re-
tant metabolic capacities that are comparable with freshly prepared cells (Zaleski et al., 1993; Steinberg et al., 1999; Silva et al., 1999). Although cryopreserved hepatocytes may be used and are suitable for drug metabolism studies, the transport of drugs within cryopreserved hepatocytes has not been reported (Kusuhara and Sugiyama, 2002). In this study, we further improved and optimized the cryopreservation methodology of rat hepatocytes to attain cells with drug transport capacities comparable with fresh cells. We furthered assessed whether a controlled freezing procedure would improve cell yield over a two-stage freezing protocol. Hengstler et al. (2000) have recently reported that a controlled slow-freezing protocol with a supercooling step minimized the release of latent heat and resulted in significant increases in viable cells after thawing. They reasoned that, as the temperature decreases and cell mixture began to freeze, crystallization would start, and the latent heat of fusion would be released resulting in the warming of the cells mixture. Since freezing and thawing are damaging to cells, these processes are major hindrances for successful cryopreservation. One way to minimize this phenomena is to supercool the freezing chamber at the moment when the cells are beginning to freeze to minimize the warming of the cell mixture as latent heat is being released. This process can be recorded by measuring the temperature of the cells in the cryovial as well as the temperature in the freezing chamber. Results from cryopreserved cells by this method clearly demonstrated that rat hepatocytes thus prepared were useful for the study of drug transport and metabolism.

Materials and Methods

Materials. ^14^C-Dextran-carboxy (6.5 mCi/ml), DMSO, polyvinyl-pyrollidone, BSA (35%), trypsin inhibitor, L15 medium Leibovitz, and Krebs-Henseleit buffer were obtained from Sigma-Aldrich (St. Louis, MO). Taurocholycholic acid (sodium salt) 54.0 mCi/mmol and Percoll were purchased from Amersham Pharmacia Biotech Inc. (Baie d’Urfe, QC). Collagenase type 2 was procured from Worthington Biochemical Corp. (Freehold, NJ). [^3^H]Estrone sulfate (specific activity, 40 Ci/mmol) and [^3^H]estradiol (specific activity, 20 Ci/mmol) was purchased from Amersham Pharmacia Biotech Inc. (Baie d’Urfe, QC). All Falcon sterile cell culture labwares were purchased from Falcon (Franklin Lakes, NJ).

Isolation of Rat Hepatocytes. All animal studies were approved by the institution’s animal care and use committee and were conducted in accordance with all applicable regulation. Rat hepatocytes were isolated from male Sprague-Dawley rats purchased from Charles River (200 Sprague-Dawley rats). Briefly, each cell aliquot was added into an Eppendorf microtube consisting of 1.4 ml final volume; sampling (125 l) layered over 10% perchloric acid (250 l), was centrifuged (14,000 rpm) for 15 s. The top layer was aspirated and a sample of incubation medium by a centrifugation method described by Fariss et al., 1985.

Cryopreservation and Thawing. Cryopreservation of rat hepatocytes was conducted by two methods. The first was one described previously (Silva et al., 1999) and involved a two-stage freezing protocol. The second method employed a controlled freezing protocol with a programamable freezer (Cryomed system from Thermo Forma, Marietta, OH). In these methods, freshly isolated hepatocytes were incubated for 30 min in Krebs-Henseleit buffer containing 15 mM glucose and 12 mM HEPES, pH 7.4, at 5 × 10^6 cells/ml and in a shaker-water bath at 37°C under an atmosphere of 95% O_2/5% CO_2. Cells were centrifuged and resuspended in cold L15-media containing 40% fetal bovine serum and 5% BSA, pH 7.4. Then a mixture containing an equal volume of cold L15-media containing 26% DMSO and 4% polyvinyl-pyrollidone was added slowly (1 ml/min). Cells were transferred into 5-ml cryogenic tubes (Corning, Palo Alto, CA) and kept on ice for 10 min. For the freezing method of Method 1, the cells were placed at −20°C for 1 h and at −70°C for another hour before being stored in liquid nitrogen (Silva et al., 1999). For Method 2, we furthered assessed whether a controlled freezing procedure would improve over the two-stage freezing protocol of Method 1. A freezing procedure controlled by a Cryomed Programmable freezer (Thermo Forma) was used. That included a slow freezing at −1°C/min until the temperature reached −7°C in the sample, which is 2°C before the expected freezing point. That was followed by a supercooling step at −60°C/min to −80°C in the chamber to rapidly adsorb the release of latent heat. Then the chamber was reheated at +40°C/min to −20°C, and the freezing process was continued at −1°C/min to −40°C in the chamber. A rapid freezing step at −10°C/min to −90°C in the chamber was done to complete the freezing Method 2. Cryotubes were then rapidly placed into a liquid nitrogen storage reservoir and stored for at least 1 week prior to thawing.

When needed, hepatocytes were rapidly thawed by immersing the cryogenic tubes in a 45°C water bath just long enough to melt the solution. Iced-cold L15-media containing 0.2% BSA was added slowly 1 to 2 ml/min to dilute the DMSO, followed with washing of the cells by gentle centrifugation at 50g. The cell pellet was then resuspended in Krebs-Henseleit buffer containing 12 mM Hepes, pH 7.4, and cell viability was assessed by the Trypan blue exclusion test. A 30% isotonic Percoll centrifugation step was performed to remove dead cell when viability was lower than 85%.

Metabolism Studies. freshly prepared or thawed, isolated rat hepatocytes (2 × 10^6 cells/ml) were preincubated at 37°C under a 95% O_2/5% CO_2 atmosphere for 15 min before the addition of substrate. 7-Hydroxy-4-(trifluoromethyl)-coumarin (7-HFC), 100 µM, to the incubation mixtures and metabolites were Parent compounds remaining in the incubation mixtures and metabolites were analyzed with a high-performance liquid chromatography/UV system equipped with a Waters 717 plus auto-sampler, a Waters 610 pump, a Waters 600S controller, and a Waters 996 photodiode array detector. The data were collected and processed by Millenium version 3.20 software (Waters, Milford, MA).

The analysis of all samples was performed using a Zorbax RX-C_18 column, 4.6 × 150 mm, and the following detection wavelengths were used: 355 nm (7-HFC), 305 nm (DFB), 250 nm (bufuralol) and 235 nm (tolbutamide). A gradient mobile phase was used, consisting initially of 85:15, 20 mM ammonium acetate in water and acetonitrile, and was brought to 50:50 in 15 min and then to 90:10 in 3 min at a flow rate of 1.0 ml/min. Under these conditions, parent compounds (7-HFC, DFB, bufuralol, and tolbutamide) eluted at 12.7, 17.4, 12.0, and 7.5 min, respectively. The main metabolites formed were glucuronide of 7-hydroxy-4-(trifluoromethy1)-coumarin, (hydroxy)-4-(4-methyl-sulfonylphenyl)-5,5-dimethyl-4-(4-methylsulfonylphenyl)-(5H)-furan-2-one (DFB, 250 µM), probes for UGT and CYP3A activity (Charet et al., 1999), respectively, were incubated with the cells for 10 min. Bufuralol and tolbutamide (100 µM), probes for CYP2D and 2C (Kronbach et al., 1987; Miners et al., 1988), respectively, were incubated with the cells for 2 h. Each incubation was stopped by addition of an equal volume of acetonitrile for protein precipitation. Parent compounds remaining in the incubation mixtures and metabolites were analyzed with an high-performance liquid chromatography/UV system equipped with a Waters 717 plus auto-sampler, a Waters 610 pump, a Waters 600S controller, and a Waters 996 photodiode array detector. The data were collected and processed by Millenium version 3.20 software (Waters, Milford, MA).

Transport Studies. Hepatocytes suspended in Krebs-Henseleit buffer at 2 × 10^6 cells/ml were preincubated in a shaker-water bath at 37°C for 30 min under an atmosphere of 95% O_2/5% CO_2. The uptake studies were initiated by the addition of radiolabeled substrate (1 µM final concentration) to the incubation mixture (1.4 ml final volume); sampling (125 µl) was performed in duplicates at specified times. The cells were rapidly separated from the incubation medium by a centrifugation method described by Faris et al., 1985. Briefly, each cell aliquot was added into an Eppendorf microtube consisting of distilled phalate (400 µl) layered over 10% perchloric acid (250 µl) and was centrifuged (14,000 rpm) for 15 s. The top layer was aspirated and a sample of the perchloric acid layer (150 µl) was removed into a scintillation vial. Subsequent to the addition of 15 ml of liquid scintillation fluid (Ready Protein; Beckman Canada, Mississauga, ON), the radioactivity was determined using a Beckman LS5000CE counter. The volume of adherent, extracellular fluid that was centrifuged with the cells was determined by incubating the cells with [^14^C]dextran-carboxy (6.5 µCi/ml), and the associated radioactivity of substrate was subtracted from the sample incubations. Data are expressed in nanomoles of compound per million of cells, after conversion of the disintegrations per minute into nanomoles from the specific activity of the sample.

Calculation and Statistics. The initial velocity was assessed by the linear portion of the plot of the amount accumulated into cells versus time. This occurred within 1 min of the uptake study. Hence, the data points (20, 40, and 60 s) were regressed to provide the initial uptake velocity. All data were
Results

Comparison of Methods 1 and 2. When the hepatocytes were precooled to 4°C and placed at −20°C for 1 h (Method 1), the release of latent heat occurred when the temperature in the cell mixture reached approximately −9°C with an increase of temperature of approximately 2°C, as shown in Fig. 1. It took 5 min for the freezing chamber to absorb the released heat. In contrast, under conditions in which the rate of freezing was controlled and the cells were supercooled prior to the freezing point (Method 2), the release of the latent heat was dramatically minimized (Fig. 1). These conditions were found to be the ones that resulted in the least increase in temperature during the freezing process.

Both methods for cryopreservation resulted in an initial high yield of cells with good viability as measured by trypan blue exclusion (Table 1). Upon further separation of viable cells from damaged ones by Percoll centrifugation, viabilities of the resulting cells increased albeit the yields decreased. This is to be expected since the Percoll centrifugation step will remove damaged cells (less dense than intact cells), but some viable cells will also be lost. Cells cryopreserved by Method 2 consistently gave a higher yield of viable cells after Percoll centrifugation compared with cells cryopreserved by Method 1 (Table 1). Furthermore, viability of the thawed hepatocytes after a 2-h incubation was not significantly different from that of freshly isolated cells incubated over the same time periods (Table 2). In contrast, thawed cells from cryopreservation with Method 1 had significantly lower viability at the end of the 2-h incubation period (Table 2). This has also been our experience with hepatocytes obtained from other species, including dog and human (results not shown). Since we were trying to obtain the highest yield of cells after thawing, we decided at this stage to favor the Method 2 over the Method 1 to characterize cryopreserved hepatocytes in transport studies.

Metabolic Studies. To ensure that cryopreserved hepatocytes retained their metabolic capacity, substrates known to be metabolized by several cytochromes P450 (bufuralol, tolbutamide and DFB and UGT (7-HFC)) were incubated with hepatocytes before and after cryopreservation. The parent remaining after a 2-h incubation period with each probe was determined in fresh hepatocytes and compared with that observed in cryopreserved cells. Although not shown, the metabolite profiles of the probes in fresh and cryopreserved cells were identical. As shown in Fig. 2, there was no significant difference in the relative metabolism of most of these probes when incubated with fresh versus thawed cryopreserved hepatocytes. Metabolism of 7-HFC was significantly decreased (p < 0.05) in cryopreserved hepatocyte compared to freshly isolated cells. About 50% of the UGT activity was lost following cryopreservation. Reports by other groups have also

### Table 1

<table>
<thead>
<tr>
<th>Method</th>
<th>After Thawing</th>
<th>After Percoll</th>
</tr>
</thead>
<tbody>
<tr>
<td>Method 1</td>
<td>78 ± 4</td>
<td>80 ± 4</td>
</tr>
<tr>
<td>Method 2</td>
<td>82 ± 2</td>
<td>87 ± 6</td>
</tr>
</tbody>
</table>

*Viability was measured by trypan blue exclusion assay immediately after thawing the cells and after a Percoll gradient to remove the dead or damaged cells, as described under Materials and Methods. Recovery was defined as the percentage of cells left relative to the number of cells initially cryopreserved. Data are expressed as mean ± S.E.M. There was no significant difference in cell survival immediately after thawing, but there was a significant difference according to the t test (P < 0.05; n = 6) (*) between the two cryopreservation methods after the Percoll step.

<table>
<thead>
<tr>
<th>Method</th>
<th>Percentage of viability (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Method 1</td>
<td>72.0 ± 3.4</td>
</tr>
<tr>
<td>Method 2</td>
<td>71.1 ± 3.6</td>
</tr>
<tr>
<td>Method 2</td>
<td>47.2 ± 5.1</td>
</tr>
</tbody>
</table>

*Isolated rat hepatocytes were cryopreserved following two different protocols: Method 1 referred to 1 h at −20°C then 1 h at −70°C, and Method 2 referred to use of the Forma Scientific freezer as described under Materials and Methods.

### Table 2

<table>
<thead>
<tr>
<th>Method</th>
<th>After Thawing</th>
<th>After Percoll</th>
</tr>
</thead>
<tbody>
<tr>
<td>Method 1</td>
<td>70.3 ± 0.3</td>
<td>57.5 ± 7.0</td>
</tr>
<tr>
<td>Method 2</td>
<td>70.5 ± 3.2</td>
<td>70.5 ± 3.2</td>
</tr>
</tbody>
</table>

*Viability was assessed by trypan blue exclusion after 2 h of incubation in Krebs-Henseleit buffer as described under Materials and Method. Data are expressed in percentage of viable cells ± S.E.M.

*Isolated rat hepatocytes were cryopreserved following two different protocols: Method 1 and Method 2 as described under Materials and Methods.

*Viability of cells from Method 1 is significantly different from that of fresh cells (p < 0.05) for n = 4.

### Metabolic Studies

Relative amount of metabolism of 7-HFC, DFB, bufuralol, and tolbutamide in freshly isolated hepatocytes (open) were compared with that observed in cryopreserved (gray) cells as described under Materials and Methods. Isolated rat hepatocytes were cryopreserved using Method 2 as described under Materials and Methods. Data are expressed as mean ± S.E.M. *, significantly different from freshly prepared cells, P < 0.05 according to the t test, n = 4.
TABLE 3
Lack of difference in uptake of various radiolabeled substrates

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Uptake Rate (nmol/min/10^6 cells)ᵃ</th>
</tr>
</thead>
<tbody>
<tr>
<td>[14C]taurocholate</td>
<td>0.15 ± 0.02</td>
</tr>
<tr>
<td>[3H]estrone Sulfate</td>
<td>0.13 ± 0.01</td>
</tr>
<tr>
<td>[3H]estradiol 17β-D-glucuronide</td>
<td>0.14 ± 0.03</td>
</tr>
</tbody>
</table>

ᵃ The rate of uptake was extrapolated from the slope of the linear portion of the curve (points of 0.15 to 1 min). No statistical difference, n = 3.

Figure 3: Effect of cryopreserving rat hepatocytes on the uptake transport of tracer concentrations of model compounds.

Cryopreserved or freshly isolated rat hepatocytes in Krebs-Henseleit buffer, pH 7.4, were incubated as described under Materials and Methods. [14C]taurocholate (A), [3H]estrone sulfate (B), and [3H]estradiol 17β-D-glucuronide (C) transport into hepatocytes was measured as described under Materials and Methods. Cells were incubated with 1 μM of compounds. At each time point an aliquot was spun through oil to pellet viable cells as described under Materials and Methods. Data are expressed as mean ± S.E.M. There is no significantly difference. n = 4 (A), n = 3 (B) and (C).

Discussion

The isolated hepatocyte system that contains intact membranes serves as an important, physiologically relevant experimental tool for uptake studies. Some work has been carried out with human hepatocytes (Olinga et al., 1998a,b) whereas rat hepatocytes are used more routinely. The preparation is facile (Moldeus et al., 1978) and the intact cells contain the entire complement of transporters, including passive permeation and transporter-assisted uptake. The incubation of drug with freshly isolated suspended hepatocytes is usually conducted over a short time-frame (usually 1 min) such that the net uptake rate is mostly influx with little efflux and is time-independent. Conventional cultured cells tend to rapidly lose hepatic transport activity (Liang et al., 1993). Recently, it has been reported that hepatocytes cultured in collagen-sandwich environment regain polarity and form bile canalicular networks (Liu et al., 1998). In this model the biliary efflux transporters such as MRPs are maintained, however, uptake transporters such as NTCP are significantly down-regulated (Liu et al., 1999). In contrast, freshly isolated hepatocytes are reported to reflect the relative uptake rates found in vivo (Sandker et al., 1994; Zhou et al., 1994). Thus, cryopreservation poses as a viable alternate procedure for the storage of human and animal hepatocytes. Cryopreserved hepatocytes have been shown to retain most of the drug-metabolizing activities (Li et al., 1999) as well as inducibility of the drug-metabolizing enzymes (Silva et al., 1999; Hengstler et al., 2000). However, use of cryopreserved hepatocytes for uptake studies has not been attempted.

We have previously described a technique for cryopreserving rat and human hepatocytes (Silva et al., 1999). Our previous, two-step freezing method (Method 1) demonstrated the importance of allowing freshly isolated hepatocytes to recover their ATP levels by preincubation before cryopreservation and the importance of allowing cryopreserved hepatocytes to recover their capacity to transport compounds across the plasma membrane. Furthermore, the transport of these compounds was temperature dependent and dose-saturating (results not shown), consistent with the mechanism of an active-transport mechanism. As an example, the \( K_m \) and \( V_{max} \) of taurocholate uptake were 36 μM and 12.3 nmol/10^6 cells/min in freshly isolated cells, and 42 μM and 11.3 nmol/10^6 cells/min in thawed cryopreserved (Method 2) cells.
bating the cells for 30 min at 37°C prior to freezing (Silva et al., 1999). The present programmed freezing protocol (Method 2) showed improved recovery (Table 1) and viability (Table 2). Moreover, similar phase I metabolic activities were observed (Fig. 2). Although UGT lost 50% activity, phase II metabolic activity was still observed in cryopreserved rat hepatocytes (Fig. 2).

More importantly, we demonstrated, for the first time that cryopreserved hepatocytes retained their ability in drug transport. To assess the ability of thawed cryopreserved hepatocytes to transport compounds, we focused on three well known substrates of uptake transporters and compared their uptake in thawed cryopreserved hepatocytes to freshly prepared hepatocytes that are shown to retain functional uptake transporter activities (Kato et al., 1999). Moreover, uptake by freshly prepared hepatocytes appears to be predictive of drug uptake in vivo in the liver (Akhteruzzaman et al., 1999; Kato et al., 1999; Abu-Zahra et al., 2000). As a starting point for investigation, the compounds chosen, taurocholate, estrone sulfate, and tauro-

References


