Rapid decrease in cellular sodium and chloride content during cold incubation of cultured liver endothelial cells and hepatocytes

Elke R. GIZEWSKI*, Ursula RAUEN*, Michael KIRSCH*, Irith REUTERS*, Herbert DIEDERICHST† and Herbert DE GROOT*‡

*Institut für Physiologische Chemie and †Institut für Arbeitsmedizin, Universitätsklinikum, Hufelandstrasse 55, D-45122 Essen, Germany

Hypothermia, as used for organ preservation in transplantation medicine, is generally supposed to lead to an intracellular accumulation of sodium, and subsequently of chloride, via inhibition of the Na^+/K^+ -ATPase. However, on studying the cellular sodium concentration of cultured liver endothelial cells using fluorescence microscopy, we found a 55% decrease in the cellular sodium concentration after 30 min of cold incubation in University of Wisconsin (UW) solution. To confirm this surprising result, we set up a capillary electrophoresis method that allowed us to determine the cellular contents of inorganic cations and of inorganic anions. Using this method we measured a decrease in the cellular sodium content from 104 ± 11 to 55 ± 4 nmol/mg of protein, accompanied by a decrease in the

chloride content from 71 ± 9 to 25 ± 5 nmol/mg of protein, after 30 min of cold incubation in UW solution. When the endothelial cells were incubated in cold Krebs–Henseleit buffer or in cold cell culture medium instead of UW solution, similar early decreases in cellular sodium and chloride contents were observed, thus excluding the possibility of the decreases being dependent on the preservation solution used. Furthermore, experiments with cultured rat hepatocytes yielded a similar decrease in sodium content during initiation of cold incubation in UW solution, so the decrease does not appear to be cell-specific either. These results suggest that, contrary to current opinion, sodium efflux predominates over sodium influx during the early phase of cold incubation of cells.

INTRODUCTION

In transplantation medicine, organs are stored at 4 °C in order to minimize hypoxic injury to the grafts during the time needed for transport and for recipient preparation [1]. However, although hypoxic injury is greatly diminished by cold storage [2,3], hypothermia itself can lead to cellular injury [4–6]. The inhibition of the Na^+/K^+ -ATPase at low temperatures, leading to the intracellular accumulation of sodium and subsequently of chloride, a process that is followed by cell swelling, is considered to be the main factor contributing to cold-induced cell injury [2,3,6]. On the basis of this hypothesis, specific organ preservation solutions have been designed in order to minimize changes in cellular ion homeostasis and in cellular volume during cold storage [1,7]. The most widely used preservation solution is University of Wisconsin (UW) solution, which contains high potassium (140 mM) and low sodium (20 mM) concentrations and the impermeable anion lactobionate instead of chloride. However, experimental data on imbalances in cellular ion homeostasis during hypothermia are scarce [8], and some cell types such as liver endothelial cells still suffer severe injury during cold storage in UW solution [1,3,9–12].

In an attempt to study such injury, we started by analysing the cellular ion concentrations of liver endothelial cells during the early stages of cold incubation in UW solution. Using digital fluorescence microscopy, we surprisingly found a pronounced decrease in the cytosolic sodium concentration, instead of the expected increase. This decrease was confirmed by means of a newly established capillary electrophoresis (CE) method, and was not confined either to UW solution or to endothelial cells.

EXPERIMENTAL

Materials

Dodecyltrimethylammonium bromide, CHAPS, probenecid,

monensin, gramicidin and fetal calf serum were obtained from Sigma (Deisenhofen, Germany). LiOH was purchased from Fluka (Neu-Ulm, Germany). BSA, insulin and penicillin were obtained from Behring Institut (Mannheim, Germany). The acetoxymethyl ester of sodium-binding benzofuran isophthalate (SBFI-AM), SBFI (tetra-ammonium salt), Pluronic F-127 and nigericin were purchased from Molecular Probes (Eugene, OR, U.S.A.). UW solution was obtained from du Pont (Bad Homburg, Germany), fibronectin from Boehringer (Mannheim, Germany) and dexamethasone from Serva (Heidelberg, Germany). Leibovitz L-15 medium and RPMI 1640 were purchased from Gibco (Eggenstein, Germany). All other chemicals were purchased from Merck (Darmstadt, Germany). Falcon cell culture dishes were obtained from Becton Dickinson (Heidelberg, Germany) and glass coverslips were from Assistent (Sondheim/Röhn, Germany).

Cell culture

Rat liver endothelial cells were isolated as described previously [11]. The cells were cultured in RPMI 1640 medium supplemented with fetal calf serum (20%, v/v), L-glutamine (2 mM), gentamycin (100 µg/ml), cefotaxime (10 µg/ml), amphotericin B (5 µg/ml) and dexamethasone (1 µM) at 37 °C in a 100% humidified atmosphere containing 5% CO_2 /95% air. Subcultures were obtained by trypsinization (0.25% trypsin in citrate saline for 5 min). Trypsin was inactivated by the addition of RPMI medium containing 20% fetal calf serum. For the fluorescence measurements, the cells were cultured on 11 cm² glass coverslips coated with fibronectin. For determining cellular ion contents, cell culture grade Petri dishes (Falcon; 3002/3003) were used [100 mm diam. for the atomic absorption spectrometry (AAS) method; 60 mm diam. for the CE method]; these dishes were chosen because they gave low ion contamination. The

Abbreviations used: UW solution, University of Wisconsin solution; KH buffer, Krebs–Henseleit buffer; AAS, atomic absorption spectrometry; CE, capillary electrophoresis; SBFI, sodium-binding benzofuran isophthalate; SBFI-AM, acetoxymethyl ester of SBFI.

‡ To whom correspondence should be addressed.

endothelial cells were used for experiments 7 days after subcultivation, by which time the cells had formed confluent monolayers.

Hepatocytes were isolated from male Wistar rats (180–220 g) as described previously [13]. Cells were seeded onto cell culture grade Petri dishes (60 mm diam.) and cultured in L-15 medium supplemented with 5% (v/v) fetal calf serum, L-glutamine (2 mM), glucose (8.3 mM), BSA (0.1%, w/v), NaHCO_3 (14.3 mM), gentamycin (50 mg/l) and dexamethasone (1 μM). At 2 h after seeding, adherent cells were washed three times with Hanks balanced salt solution and supplied with fresh medium. Hepatocytes were used for experiments 20–24 h after isolation.

Incubations

Cells were washed three times with Hanks balanced salt solution (137 mM NaCl, 5.4 mM KCl, 1 mM CaCl_2 , 0.5 mM MgCl_2 , 0.4 mM KH_2PO_4 , 0.4 mM MgSO_4 , 0.3 mM Na_2HPO_4 , 20 mM Hepes; 37 °C) and then covered with cold (4 °C) UW solution (20 mM Na^+ , 140 mM K^+ , 5 mM Mg^{2+} , 25 mM H_2PO_4^- , 5 mM SO_4^{2-} , 100 mM lactobionate, 30 mM raffinose, 50 g/l hydroxyethyl-starch, 1 mM allopurinol, 5 mM adenosine, 3 mM glutathione, pH 7.4; before use, the solution was supplemented with 40 units/l insulin, 16 mg/l dexamethasone and 2×10^5 units/l penicillin), Krebs–Henseleit (KH) buffer (115 mM NaCl, 25 mM NaHCO_3 , 5.9 mM KCl, 1.2 mM MgCl_2 , 1.2 mM NaH_2PO_4 , 1.2 mM Na_2SO_4 , 2.5 mM CaCl_2 , 20 mM Hepes) or cell culture medium (supplemented as described above). Incubations with KH buffer or cell culture medium were placed in an air-tight vessel flushed with 5% CO_2 /21% O_2 /74% N_2 . Incubations with UW solution were exposed to air. KCN (final concentration 1 mM) was added to some incubations in order to simulate hypoxic conditions.

For viability controls, UW solution and KH buffer contained Trypan Blue at a final concentration of 85 μM . Cell injury was assessed by the uptake of the vital dye Trypan Blue and by the release of cytosolic lactate dehydrogenase.

Analysis of sodium concentration

The cytosolic sodium concentration was measured by digital fluorescence microscopy using the Na^+ -sensitive fluorescent dye SBFI. The coverslips containing the endothelial cell monolayers were transferred to a Pentz chamber (aluminium/Teflon with a heat-conductive bottom; manufactured at the workshop of the Medical School). The dye was loaded into the cells as the membrane-permeable acetoxymethyl ester, and retention of the dye in endothelial cells was improved by the addition of probenecid [14]. For loading, the cells were incubated for 180 min at 37 °C in the dark in KH buffer containing 1.2 mM probenecid, 0.09% Pluronic F-127 and 10 μM SBFI-AM. After this incubation the coverslips were rinsed three times with KH buffer and incubated for another 30 min in the dark.

Fluorescence measurements were performed using an inverted microscope (Axiovert 135 TV; Zeiss, Oberkochen, Germany) equipped with the Attofluor imaging system (Atto Instruments, Rockville, MD, U.S.A.). The required temperature was maintained using a liquid-cooled aluminium microscope stage (Zeiss) which was connected via three-way taps to a cryostat set at 0 °C and a water bath set at 38 °C (2219 Multitemp II; Pharmacia Biosystems, Freiburg, Germany). These settings resulted in temperatures of 4 ± 1 °C and 37 ± 1 °C in the supernatant, as assessed using a digital thermometer with a filamentous probe.

The cytosolic free sodium concentration was measured by ratio imaging of SBFI fluorescence using excitation wavelengths of 340 and 380 nm and an emission wavelength of 500–530 nm. *In situ* calibration of the sodium concentration was performed by

the method of Kawanishi et al. [15] with minor modifications. Lactobionate was used as the impermeant anion. Sodium-containing and potassium-containing solutions were prepared for each temperature, containing: 120 mM lactobionic acid, 120 mM potassium or 120 mM sodium, 25 mM Hepes, 1.2 mM CaCl_2 , 0.6 mM MgCl_2 , gramicidin (4 μM for 37 °C and 10 μM for 4 °C), 10 μM monensin and 10 μM nigericin; the pH was adjusted to 7.4 using either NaOH or KOH. Solutions containing 0–120 mM sodium were prepared by mixing these two solutions as appropriate. The same experimental approach was used to determine the apparent K_d for SBFI in ionophore-treated cells. The apparent K_d was then calculated as described by Jung et al. [16].

Measurement of the dissociation constant of SBFI in a cell-free system

To determine the dissociation constants of SBFI for sodium at 37 °C and at 4 °C, fluorescence measurements were performed using an Aminco–Bowman spectrophotofluorimeter as described by Minta et al. [17]. SBFI free acid (10 μM) was added to cell-free Hepes-buffered solution (20 mM, pH 7.4) containing different sodium concentrations (0–35 mM). The fluorescence was excited at 334 and 380 nm, and a photomultiplier read the emission at 520 nm. The K_d was then calculated according to standard methods.

Analysis of the cellular ion content

Immediately after the addition of UW solution or KH buffer (0 h), and after 0.5, 1 and 3 h of cold incubation, the cells were washed three times with ice-cold sucrose solution (310 mM, pH 7.4; pH adjusted with LiOH). The cells were then lysed with 50 mM CHAPS (30 min at 37 °C). An aliquot of the lysate was used to determine the protein content of the cells, and the remainder was used for CE analysis.

The water used was generated by an 18 M Ω Milli-Q laboratory water purification system (Millipore, Eschborn, Germany), and is referred to below as 18 M Ω water. As alternative methods for cell lysis, 6.5% HNO_3 , 2% Triton X-100, 20 mM CHAPS, 3% Pluronic F-127 and freeze–thaw cycles (18 M Ω water; freeze at -70 °C; thaw at $+37$ °C; three cycles) were used.

The CE system employed was the P/ACE 5000 instrument from Beckman (Munich, Germany). The system comprised a selectable fixed-wavelength UV detector and a 0–30 kV high-voltage built-in power supply. Fused silica capillaries (75 μm inner diameter and 57 cm long) were obtained from Beckman. Standard Beckman glass vials were used for the carrier electrolytes, standards and samples. Gold software (Beckman) was used for system control and data handling. The temperature was set at 23 °C. The capillary was rinsed with 0.1 mM LiOH (304 Pa), 18 M Ω water (304 Pa) and separation buffer (304 Pa). The samples were introduced by pressure injection (7.6 Pa) for 5 s.

Carrier electrolytes and standards were prepared using 18 M Ω water. Sodium, potassium and magnesium standards were prepared from NaCl, KI and $\text{Mg}(\text{NO}_3)_2$ respectively, and chloride, sulphate, carbonate and phosphate standards from NaCl, MgSO_4 , KHCO_3 and KH_2PO_4 respectively. They were dissolved in the equivalent detergent or water as appropriate. Buffer solutions were filtered, and standards and samples were centrifuged (5 min; 900 g).

The indirect detection mode was used to detect non-absorbing inorganic ions [18]. For the detection of anions, sodium dichromate at a concentration of 5 mM was used as background electrolyte, and 0.5 mM dodecyltrimethylammonium bromide was added as an electro-osmotic flow-modifier to reverse the

Table 1 Effect of cooling on SBFI fluorescence and the cytosolic sodium concentration

After loading with SBFI-AM, rat liver endothelial cells were incubated either at 37 °C in cell culture medium or at 4 °C in UW solution for 30 min. The ratio of the fluorescence (emission 500–530 nm) after excitation at 340 and 380 nm was determined using a digital imaging system and the sodium concentrations were determined by *in situ* calibrations at the two temperatures using extracellular solutions of different sodium concentration and the ionophores monensin, nigericin and gramicidin. The apparent dissociation constant (apparent K_d) was determined in ionophore-treated cells in independent experiments. The dissociation constant of the free dye was determined in a cell-free system. * Significantly different from value at 37 °C ($P < 0.05$).

Temperature (°C)	SBFI ratio fluorescence	Sodium concn. (mM)	Apparent K_d (ionophore-treated cells) (mM)	K_d in cell-free system (mM)
37	1.03 ± 0.01	20 ± 1	24	19
4	1.04 ± 0.02	9 ± 1*	12*	7*

direction of the flow. The pH was adjusted to 8.0 with H₂SO₄ (10%). After injection, a voltage of 25 kV at reversed polarity was applied. The anions were monitored at a wavelength of 254 nm. To detect cations, 10 mM imidazole at pH 6.0 (pH adjusted with H₂SO₄) was used as the background electrolyte. The cations were separated by applying a voltage of 20 kV and monitored at a wavelength of 214 nm. The positions of the peaks and peak areas in the cell lysates were confirmed by standard additions.

A standard method for analysing the cation content of cells by AAS (SpectraA Varian and PU 9000 Philips; Unicam, Offenbach, Germany) was used as a reference method [19–22]. To analyse the cation content of hepatocytes, only the latter method was used.

Measurement of protein content

The protein content of the HNO₃ lysates was measured using the biuret test of Bergmeyer et al. [23], modified by using nitric acid instead of trichloroacetic acid. When detergents were used to lyse the cells, the biuret test was modified such that 5 ml of biuret solution, already containing the NaOH usually used to dissolve the protein pellet, was added to a 1 ml aliquot of the detergent cell lysate. BSA, dissolved in the detergent, was used as standard.

Statistics

All experiments were performed in duplicate and repeated between two and seven times. Data are expressed as means ± S.D. Data obtained from two groups were compared by Student's *t* test [24]. A *P* value of < 0.05 was considered significant.

RESULTS

When cultured liver endothelial cells were cooled from 37 °C to 4 °C in UW solution, digital fluorescence microscopy suggested a rapid decrease in the cytosolic sodium concentration (Table 1). Although the 340/380 nm ratio remained at 1.04 during cooling, this ratio corresponded to a concentration of 20 mM at 37 °C and of 9 mM at 4 °C, as determined by *in situ* calibration. The different *in situ* calibration curves of the dye at the two temperatures mainly result from the altered K_d of the fluorescence marker SBFI at 4 °C: the K_d , as obtained using a cell-free system, was 19 mM at 37 °C (a value that fits well with that reported by Minta and Tsien [25]) and 7 mM at 4 °C. Although the apparent K_d of SBFI in ionophore-treated cells was slightly higher than in the cell-free system (a finding that has also been reported by other groups [26,27], and might be explained by the known effects of cytosolic viscosity, ionic strength and pH on fluorescent probes [14,27]), it showed the same decrease as the temperature decreased (Table 1). The results of the fluorescence microscopy experiments thus pointed to a 55% decrease in the free cytosolic sodium concentration after 30 min of cooling. However, as uncertainties remained with regard to the calibration of the fluorescent dye, e.g. the effectiveness of the ionophores at 4 °C, we felt that it was necessary to confirm this unexpected, massive, loss of sodium by applying a second method. As a complementary method we chose to measure the cellular ion content (which also gives an estimation of the net ion movements across the plasma membrane independent of cellular volume changes). We decided to use CE instead of AAS for these measurements, as CE also allows the determination of anion concentrations.

When the cells were lysed with 6.5% nitric acid, as used when determining the cellular cation content by AAS [22], inconstant currents were unavoidable during separation of the cations by CE. Among a variety of detergents, CHAPS at 50 mM proved to be the best alternative; it led to complete cell lysis as judged by comparison with the standard method, whereas CHAPS concentrations of less than 30 mM or the use of three freeze–thaw cycles did not lead to complete disruption of the cells (Table 2). It was

Table 2 Cation and anion contents of liver endothelial cells as analysed by CE after the application of different methods of cell lysis

The cellular cation contents measured by CE are compared with the results obtained using a standard AAS method. Values are means ± S.D.; *n* = 10. *Significantly different from the value obtained by the standard method of lysis with 6.5% HNO₃ and AAS analysis.

Method of cell lysis	Method of analysis	Cellular ion content (nmol/mg of protein)					
		Potassium	Sodium	Magnesium	Chloride	Sulphate	Phosphate
6.5% HNO ₃	AAS	622 ± 92	98 ± 21	43 ± 21	–†	–†	–†
50 mM CHAPS	CE	611 ± 38	97 ± 10	44 ± 12	72 ± 8	15 ± 3	274 ± 14
2% Triton X-100	CE	601 ± 26	–‡	44 ± 6	71 ± 5	13 ± 3	267 ± 16
20 mM CHAPS	CE	570 ± 67*	97 ± 19	38 ± 10*	66 ± 8	12 ± 4	231 ± 8
3% Pluronic F-127	CE	552 ± 59*	–‡	37 ± 4*	–§	–§	–§
Freeze–thaw	CE	471 ± 10*	104 ± 13	35 ± 2*	63 ± 15	11 ± 9	204 ± 15

† Anion analysis with AAS at the concentration used is not possible.

‡ Triton and Pluronic contain sodium in excess of 200 nmol/ml.

§ Pluronic contains chloride in excess of 100 nmol/ml.

Figure 1 Separation of inorganic ions in cell lysates using CE

Cultured rat liver endothelial cells were quickly washed with ice-cold 310 mM sucrose solution and lysed with 50 mM CHAPS (30 min). After centrifugation at 900 *g* (10 min) to remove particulate material, the detergent sample was introduced by hydrostatic injection (5 s, 7.6 Pa) into a fused silica capillary (75 μm inner diameter; 57 cm long). The sample was (a) run at 20 kV using an imidazole buffer (pH 6.0, 10 mM) and the absorbance was monitored at 214 nm (indirect detection mode) for the detection of cations, or (b) run at 25 kV (reversed polarity) using a chromate buffer (pH 8.0, 5 mM supplemented by 0.5 mM dodecyltrimethylammonium bromide) and the absorbance was monitored at 254 nm (indirect detection mode) for the detection of anions.

not possible to analyse the sodium content of the samples using Triton or Pluronic, because these detergents are not pure enough in terms of sodium concentration.

The electropherograms of a representative sample are shown in Figure 1(a) (cations) and Figure 1(b) (anions). The peaks were present at the same positions when using cell lysates as when standards were added. Standard curves were linear in the range from 4 nmol/ml to 6 $\mu\text{mol/ml}$. The detection limit was 4 nmol/ml

Figure 2 Sodium and chloride content of cultured liver endothelial cells after initiation of cold incubation in UW solution

Rat liver endothelial cells, which had been cultured at 37 °C in RPMI medium, were washed three times with warm Hanks balanced salt solution followed by the addition of cold (4 °C) UW solution. Cells were then either lysed immediately (0 h) or incubated at 4 °C in UW solution in the absence or in the presence of cyanide (final concentration 1 mM) for different time periods. After washing with 310 mM sucrose and lysis with 50 mM CHAPS, the ion content was analysed by CE. The time courses of changes in the cellular sodium content (upper panel) and the cellular chloride content (lower panel) during 3 h of cold incubation are shown. Values are means $\pm$ S.D. of six experiments.

for both anions and cations. The ion concentrations of the final washing step were found to be less than 2.5 $\mu\text{mol/ml}$ in 10 ml, corresponding to extracellular contamination of less than 1.5 nmol/ml (less than 0.5 nmol/mg of protein). As the carbonate concentration in the sample decreases with storage time, no quantitative analysis of this ion was carried out. In determining cellular calcium content, complete disruption of cellular organelles is crucial. As we did not optimize cell lysis in this respect, we did not integrate the calcium peaks.

On analysing the ion content of the cultured liver endothelial cells at 0 h, i.e. immediately after the addition of cold UW solution, we measured a sodium content of 104 ± 11 nmol/mg of protein. This, and the values for the other ions (potassium, 626 ± 40 nmol/mg of protein; magnesium, 44 ± 7 nmol/mg; chloride, 71 ± 9 nmol/mg; sulphate, 14 ± 3 nmol/mg; phosphate, 268 ± 15 nmol/mg), were equal to the contents found after washing the cells with sucrose solution before exposing them to UW solution (cf. Table 2), i.e. there was no contamination by the highly viscous UW solution. After 30 min of cold incubation in UW solution, the sodium content had decreased by about

Table 3 Decrease in the cellular sodium content of liver endothelial cells after initiation of cold (4 °C) incubation

The cellular sodium content of cultured liver endothelial cells after 0 and 30 min of cold incubation in UW solution, KH buffer or RPMI medium was analysed by means of CE and AAS (values in parentheses). The cells were incubated at 4 °C in these solutions in the absence or in the presence of cyanide (final concentration 1 mM). Values are means $\pm$ S.D. of eight experiments. *Significantly different from initial values ($P < 0.05$).

Incubation solution	Sodium content (nmol/mg of protein) after:	
	0 min	30 min
UW	104 $\pm$ 11 (104 $\pm$ 12)	55 $\pm$ 4* (49 $\pm$ 5)*
UW + KCN	106 $\pm$ 16 (109 $\pm$ 12)	53 $\pm$ 4* (43 $\pm$ 7)*
KH	105 $\pm$ 5 (98 $\pm$ 5)	57 $\pm$ 5* (47 $\pm$ 4)*
KH + KCN	100 $\pm$ 5 (103 $\pm$ 12)	57 $\pm$ 4* (52 $\pm$ 4)*
RPMI	104 $\pm$ 5 (-) [†]	55 $\pm$ 7* (-) [†]
RPMI + KCN	102 $\pm$ 6 (-) [†]	55 $\pm$ 8* (-) [†]

[†] Values not measured by AAS.

50 nmol/mg of protein, i.e. by half (Figure 2, upper panel). This decrease occurred under normoxic control conditions as well as in the presence of cyanide (chemical hypoxia). The same results were obtained when AAS was used for analysis instead of CE (Table 3). During the following 2 h the sodium content under normoxic conditions increased, whereas in the presence of cyanide the sodium content stayed at the lower level (Figure 2, upper panel). In contrast with the sodium content, there were no significant changes in the magnesium or potassium content of the endothelial cells after 3 h of cold incubation in UW solution, and the loss of viability during this time remained below 3 % (results not shown).

The chloride content of the cells incubated in cold UW solution showed similar changes to the sodium content; there was also a decrease of about 50 nmol/mg of protein within 30 min (Figure 2, lower panel). This decrease was independent of the presence of cyanide. Like the sodium content, the chloride content increased during the subsequent 2 h under normoxic control conditions but stayed at the lower level when the cells were incubated in cyanide-containing solution. The sulphate and phosphate contents did not show any significant changes after 3 h of storage at 4 °C in UW solution.

Similar results to those observed in UW solution were obtained when the endothelial cells were incubated in cold KH buffer: rapid initial decreases in the cellular sodium (Figure 3, upper panel) and chloride (Figure 3, lower panel) contents were again found in this saline solution in the presence of physiological extracellular ion concentrations. Here, too, there were no significant changes in the cellular contents of potassium, magnesium, sulphate and phosphate. Compared with the incubations in UW solution, we found more rapid increases in the cellular sodium and chloride contents in the incubations containing KCN during prolonged hypothermia.

Similarly, cooling the endothelial cells in serum-containing cell culture medium yielded a decrease of about 50 nmol/mg of protein in the cellular sodium content (Table 3). The chloride

Figure 3 Sodium and chloride content of cultured liver endothelial cells after initiation of cold incubation in KH buffer

Rat liver endothelial cells, which had been cultured at 37 °C in RPMI medium, were washed three times with warm Hanks balanced salt solution, following which cold (4 °C) KH buffer was added to the cells. Cells were then either lysed immediately (0 h) or incubated at 4 °C in KH buffer in the absence or in the presence of cyanide (final concentration 1 mM) for different time periods. After washing with 310 mM sucrose and lysis with 50 mM CHAPS, the ion content was analysed by CE. The time courses of changes in the cellular sodium content (upper panel) and the cellular chloride content (lower panel) during 3 h of cold incubation are shown. Values are means $\pm$ S.D. of four experiments.

content decreased from 74 ± 6 to 36 ± 8 nmol/mg of protein after 30 min of incubation, and no changes occurred in the contents of the other ions.

When cultured hepatocytes were incubated in cold UW solution, the cellular cation contents showed similar alterations to those found in endothelial cells: after 30 min of cold incubation, a decrease of more than 40 % in the sodium content was measured (0 min, 99 ± 10 nmol/mg of protein; 30 min, 56 ± 13 nmol/mg of protein). Again, there were no significant alterations in the cellular contents of potassium and magnesium (the anion contents in this cell type were not measured).

DISCUSSION

In transplantation medicine, organs are cooled during storage in order to decrease the energy consumption of the non-perfused organ. Although lowering the temperature effectively delays the development of energy deficiency (and also delays subsequent steps of energy-deficiency-induced injury) [2,6,28], hypothermia is thought to lead to a disturbance of cellular ion homeostasis,

primarily via inhibition of the Na^+/K^+ -ATPase [6]. This is believed to result in an increase in intracellular sodium and subsequently water, leading to cellular swelling. So far, the relatively scarce experimental data have confirmed this hypothesis. During cold incubation of erythrocytes in cell culture medium, passive sodium influx and passive potassium efflux have been measured [29], and cold incubation of umbilical vein endothelial cells in cell culture medium or heparinized sodium acetate solution has been described to lead to an increased sodium concentration, but with no change in the chloride concentration [8]. The morphological changes to endothelial cells during cold incubation were interpreted as an increase in volume [10].

However, when we measured the cytosolic sodium concentration during the incubation of cultured liver endothelial cells in cold UW solution, digital fluorescence microscopy suggested not the expected increase but, on the contrary, a marked decrease in the sodium concentration (Table 1). As this decrease in the cytosolic sodium concentration contradicts the common hypothesis [1,2,6,29], a second experimental approach appeared necessary to confirm our results.

To study cellular cation concentrations, AAS is most commonly employed [19,20,22,30]. However, CE was introduced some years ago for the analysis of cations and anions in, for example, milk and water [31,32]. In contrast with AAS, CE should thus also allow the determination of changes in the cellular concentrations of inorganic anions that were previously difficult to assess [33–38]. Therefore we decided to adapt the existing CE methods [32,39,40] to the determination of ion concentrations in cell lysates.

The standard method of cell lysis used in conjunction with AAS, i.e. lysis with 6.5% nitric acid, was problematic for CE because of high sample conductivity, but lysis with the zwitterionic detergent CHAPS at 50 mM gave results comparable with those obtained using the standard AAS method (Table 2). The advantages of this CE method are the short separation time and low sample volume (injection volume 30 or 60 nl for cations and anions respectively). Just two runs with one sample are necessary to measure the cation and anion concentrations respectively. We used pressure injection of the samples instead of the more sensitive electrokinetic injection, because electrokinetic injection causes distortions in the way that changes in the amount of one ion influence the injection of the others. If one relates the measured ion contents to a cell volume of 4.5 $\mu\text{l}/\text{mg}$ of protein, as has been reported for aortic endothelial cells [20], our results would yield a cellular sodium concentration of 20 mM, a cellular potassium concentration of 140 mM, a cellular magnesium concentration of 10 mM (total, not free, magnesium), a cellular chloride concentration of 20 mM and a cellular phosphate concentration of 60 mM, i.e. the values obtained appear fairly realistic.

On analysing the sodium content of the liver endothelial cells during the early phase of cold incubation in UW solution, we found a 55% decrease (Figure 2, upper panel). This means that either the intracellular concentration of sodium decreases or, if the concentration does not change, there would have to be a massive loss of cellular volume. As the content of the main intracellular cation potassium remained nearly constant during cold incubation, large volume changes have to be excluded. Thus the 55% decrease in the cellular sodium content in the presence of an almost unaltered potassium content fits well with the 55% decrease in the sodium concentration described above. The additional initial decrease in the cellular chloride content which, amounting to 50 nmol/mg of protein, was equimolar with the decrease in the cellular sodium content, suggests that chloride is

the accompanying anion. The surprising decrease in the cellular sodium content was not peculiar to liver endothelial cells, but was also observed in hepatocytes, although some other cell types might react differently [41].

Modern preservation solutions, and especially UW solution, were designed to counteract the alterations in cellular ion homeostasis normally induced by hypothermia. Therefore the unexpected decrease in cellular sodium and chloride contents might be the result of a very efficient, 'overshooting' prevention of the expected increases by the preservation solution with its high potassium and low sodium concentrations. To address this point, we incubated cells in cold KH buffer and in cold cell culture medium. There were similar initial decreases in the cellular sodium and chloride contents when the cells were cooled in KH buffer (Figure 3) or cell culture medium (Table 3). Thus the important factor does not appear to be the specific ion composition of the UW solution but the low temperature itself. It has been suggested that hypothermia leads to changes in the plasma membrane from a liquid/crystalline phase to a gel phase [42]. Furthermore, the activity of ion transporters has been shown to decrease [6,28]. The decline in ATP levels provoked by inhibition of the mitochondrial respiratory chain should, in addition, aggravate the inhibition of the Na^+/K^+ -ATPase. All of these hypothermia-induced alterations should lead to an increase in the cellular sodium concentration. In contrast, our results suggest that, during the early phase of cooling, the efflux of sodium and chloride is greater than the influx, i.e. sodium must be actively transported out of the cell. This would mean that decreases in the activity of sodium transporters (mediating inward transport) must be greater or more rapid than the decrease in Na^+/K^+ -ATPase activity during cooling (even in the presence of cyanide). Whether these differences in activity result from different temperature-sensitivities of the transporters, or whether cellular signalling pathways are involved, is unclear at present.

Whereas during the early period of cooling cells lost sodium and chloride, during prolonged hypothermia the cellular contents of these ions rose again under most experimental conditions; only when cells were incubated in UW solution in the presence of cyanide did the sodium and chloride contents remain at the lower levels. Thus the alterations observed during prolonged hypothermia in solutions of physiological ion composition (KH buffer and cell culture medium) are in line with other studies [29], which showed sodium influx and potassium efflux at a later time, and with the generally accepted concepts [6]. The same holds true for the enhancement of the late rise in cellular sodium observed during cold incubation in these solutions in the presence of the mitochondrial respiratory chain inhibitor cyanide. The differing results in UW solution, with cyanide inhibiting instead of enhancing the late increases in sodium and chloride contents, might be related to differences in the mechanism of injury in UW solution compared with KH buffer. Whereas the addition of cyanide to KH buffer aggravated injury, the injury in UW solution could be largely inhibited by the addition of cyanide, a feature we interpreted as energy-dependence, although the exact mechanism of this energy-dependent, free-radical-mediated injury is not yet clear [11,43]. While the early decrease in the cellular sodium content shown here was very unexpected with regard to the literature, at present it cannot be excluded that the later increase is more closely related to the pathogenesis of injury during cold incubation in all or some solutions. The relationship of the observed alterations in cellular ion homeostasis to the pathogenesis of preservation injury appears to be complex, and requires further study.

In conclusion, we have observed a massive decrease in cellular sodium and chloride contents within the first 30 min of cooling

of cultured liver endothelial cells and hepatocytes. We have also established a method that allows one to analyse not only the cation content but also the anion content of cultured cells.

We thank Dr. B. von Saan for her help with the AAS measurements. This work was supported by the Deutsche Forschungsgemeinschaft (Gr 815/6-1).

REFERENCES

- Lemasters, J. J., Bunzendahl, H. and Thurman, R. G. (1995) Liver Transplant. Surgery **1**, 124–138
- Blankensteijn, J. D. and Terpstra, O. T. (1991) Hepatology **13**, 1235–1247
- Clavien, P. A., Harvey, P. R. C. and Strasberg, S. M. (1992) Transplantation **53**, 957–978
- Kruuv, J., Glofcheski, D. J. and Lepock, J. R. (1995) Cryobiology **32**, 182–190
- Hansen, T. N., Dawson, P. E. and Brockbank, K. G. M. (1994) Cryobiology **31**, 101–106
- Hochachka, P. W. (1986) Science **231**, 234–241
- Belzer, F. O. and Southard, J. H. (1988) Transplantation **45**, 673–675
- Larsen, T., Solberg, S., Johansen, R. and Jorgensen, L. (1988) Scand. J. Clin. Lab. Invest. **48**, 565–571
- Caldwell-Kenkel, J. C., Currin, R. T., Tanaka, Y., Thurman, R. G. and Lemasters, J. J. (1991) Hepatology **13**, 83–95
- McKeown, C. M. B., Edwards, V., Phillips, M. J., Harvey, P. R. C., Petrunka, C. N. and Strasberg, S. M. (1988) Transplantation **46**, 178–191
- Rauen, U., Hanssen, M., Lauchart, W., Becker, H. D. and de Groot, H. (1993) Transplantation **55**, 469–473
- Rauen, U., Hintz, K., Hanssen, M., Lauchart, W., Becker, H. D. and de Groot, H. (1993) Transplant. Int. **6**, 218–222
- de Groot, H. and Brecht, M. (1991) Biol. Chem. Hoppe-Seyler **372**, 35–41
- Harootunian, A. T., Kao, J. P. Y., Eckert, B. K. and Tsien, R. Y. (1989) J. Biol. Chem. **264**, 19458–19467
- Kawanishi, T., Nieminen, A.-L., Herman, B. and Lemasters, J. J. (1991) J. Biol. Chem. **266**, 20062–20068
- Jung, D. W., Apel, L. M. and Brierley, G. P. (1992) Am. J. Physiol. **262**, C1047–C1055
- Minta, A., Harootunian, A. T., Koo, J. P. Y. and Tsien, R. Y. (1987) J. Cell Biol. **105**, 492
- Yeung, E. S. and Kuhr, W. G. (1991) Anal. Chem. **63**, 275A–282A
- Welz, B. (1985) Atomic Absorption Spectrometry, VCH, Weinheim
- O'Neill, W. C. and Klein, J. D. (1992) Am. J. Physiol. **262**, C436–C444
- Navas, J. P., Anderson, W. and Marsh, J. D. (1990) Am. J. Physiol. **259**, H333–H339
- Brock, T. A., Brugnara, C., Canessa, M. and Gimbrone, M. A. (1986) Am. J. Physiol. **250**, C888–C895
- Bergmeyer, H. U., Bernt, E., Gawehn, K. and Michal, G. (1974) in Methoden der enzymatischen Analyse (Bergmeyer, H. U., ed.), pp. 184–188, Verlag Chemie, Weinheim
- Armitage, P. (1971) Statistical Methods in Medical Research, Blackwell Scientific Publications, Oxford
- Minta, A. and Tsien, R. Y. (1989) J. Biol. Chem. **264**, 19449–19457
- Levi, A. J., Lee, C. O. and Brooksby, P. (1994) J. Cardiovasc. Electrophysiol. **5**, 241–257
- Negulescu, P. A. and Machen, T. E. (1990) Methods Enzymol. **192**, 38–81
- Churchill, T. A. and Fuller, B. J. (1995) Transplantation **59**, 904–907
- Kimzey, S. L. and Willis, J. S. (1971) J. Gen. Physiol. **58**, 634–649
- Murphy, E., Aiton, J. F., Horres, C. R. and Lieberman, M. (1983) Am. J. Physiol. **245**, C316–C321
- Schmitt, M., Saulnier, F., Malhautier, L. and Linden, G. (1993) J. Chromatogr. **640**, 419–424
- Jandik, P. and Bonn, G. (1993) Capillary Electrophoresis of Small Molecules and Ions, VCH, New York
- Levinson, C. (1992) J. Membr. Biol. **126**, 277–284
- Verkman, A. S. (1990) Am. J. Physiol. **259**, C375–C388
- Chao, A. C., Dix, J. A., Sellers, M. C. and Verkman, A. S. (1989) Biophys. J. **56**, 1071–1081
- Jensen, B. S., Jessen, F. and Hoffmann, E. K. (1993) J. Membr. Biol. **131**, 161–178
- Hall, S. R., Sigee, D. C. and Beesley, J. E. (1992) Scanning Microsc. **6**, 753–763
- Glauert, A. M. (1977) in X-Ray Microanalysis in the Electron Microscope (Chandler, J. A., ed.), pp. 500–507, North Holland Publishing Co., Amsterdam
- Quang, C. and Khaledi, M. G. (1994) J. Chromatogr. **659**, 459–466
- Weston, A., Brown, P. R., Jandik, P., Heckenberg, A. L. and Jones, W. R. (1992) J. Chromatogr. **608**, 395–402
- Knerr, S. M. M. and Liebermann, M. (1993) J. Mol. Cell. Cardiol. **25**, 277–288
- Crowe, J. H., Hoekstra, F. A., Crowe, L. M., Anchordoguy, T. J. and Drobins, E. (1989) Cryobiology **26**, 76–84
- Rauen, U., Elling, B., Gizewski, E. R., Korth, H.-G., Sustmann, R. and de Groot, H. (1997) Free Radicals Biol. Med. **22**, 17–24