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Metabolic viability and pharmaco-toxicological reactivity of cryopreserved human precision-cut renal cortical slices

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Abstract

We have tested the suitability of cryopreserved human precision-cut renal cortical slices for metabolic and pharmaco-toxicological studies. The viability of these slices and their pharmaco-toxicological reactivity were assessed using intracellular ATP and protein contents, lactate dehydrogenase (LDH) leakage, lactate and glutamine metabolism and the ammoniagenic effect of valproate. Despite a decrease in ATP and protein contents when compared with those of fresh slices, cryopreserved slices did not show any LDH leakage and retained the capacity to metabolize glutamine and lactate. Glutamine removal and ammonia, lactate and alanine production were similar in fresh and cryopreserved slices; by contrast, cryopreserved slices accumulated more glutamate as a result of decreased flux through glutamate dehydrogenase which catalyses an oxygen-dependent reaction. Valproate markedly and similarly stimulated glutamine metabolism in fresh and cryopreserved slices. Cryopreservation did not alter lactate removal but inhibited lactate gluconeogenesis. In conclusion, these results demonstrate that, although their mitochondrial oxidative metabolism seems to be diminished, cryopreserved human precision-cut renal cortical slices remain metabolically viable and retain the capacity to respond to the ammoniagenic effect of valproate. Thus, this experimental model may be helpful to optimize the use of human renal tissue for metabolic and pharmaco-toxicological studies.

1. Introduction

It is well-established that the human kidney takes up glutamine from the circulating blood under various physiological and pathophysiological situations (Owen and Robinson, 1963; Wahren and Felig, 1975; Tizianello et al., 1980; Brundin and Wahren, 1994; Meyer et al., 1998; Cersosimo et al., 2000). The renal metabolism of glutamine, the major precursor of the ammonium ions excreted in the urine, is of central importance for the regulation of systemic acid-base homeostasis (for reviews, see Curthoys and Watford, 1995; Nissim, 1999; Welbourne et al., 2001). Despite this, the mechanisms regulating or disturbing the human renal metabolism of glutamine, which cannot be studied in vivo, remain poorly understood. To our knowledge, only a few studies have been devoted to glutamine metabolism in human renal cells in vitro (Watford et al., 1980; Nissim and States, 1989; Martin et al., 1990; Nakada et al., 1994; Conjard et al., 2001). This is most likely due to the limited availability of viable human kidney cells for in vitro studies. Thanks to the precious collaboration of surgeons and pathologists, we have performed in this laboratory a number of metabolic studies using isolated human proximal tubules or segments (Martin et al., 1990; Conjard et al., 2001; Baverel et al., 1979; Michoudet and Baverel, 1987; Fouque et al., 1996; Dubourg et al., 2001). But the latter studies were completed very slowly because the amount of cortical tissue available, and therefore, the experimental protocol, greatly varied from one experiment to another. In certain experiments, the amount of human renal tissue provided exceeded our needs. In an attempt to optimize the use of human renal tissue when available in large quantities, we have tested in this study the possibility of using cryopreservation. For this, we have used precision-

Abbreviations: ATP, adenosine triphosphate; DMSO, dimethylsulfoxide; LDH, lactate dehydrogenase.

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cut slices for which there is a renewed interest particularly for hepatotoxicity and, to a lesser extent, for nephrotoxicity studies (for reviews, see Parrish et al., 1995; Bach et al., 1996; Lerche-Langrand and Toutain, 2000); indeed, such slices, which maintain cell heterogeneity and tissue architecture and whose preparation does not require the use of proteolytic enzymes, retain their metabolic viability for at least 24 h whereas kidney tubules isolated with collagenase remain metabolically viable for only some hours (Chauvin et al., 1996).

In the present study, human precision-cut renal cortical slices, both freshly prepared on the one hand, and fast-frozen and thawed on the other hand, were incubated in the presence of glutamine as substrate and their glutamine metabolism (see Fig. 1) has been characterized. Their pharmaco-toxicological reactivity to valproate, a widely used antiepileptic drug and a hyperammonemic agent (Simon and Penry, 1975; Coulter and Allen, 1980), has also been studied; it is indeed well-established that valproate is a stimulator of human renal glutamine metabolism both in vivo (Warter et al., 1984) and in vitro (Martin et al., 1990).

The results obtained clearly indicate that the fast-freezing and thawing procedure used preserves in a satisfactory manner the metabolic viability and pharmaco-toxicological reactivity of human precision-cut renal cortical slices at least when glutamine metabolism is concerned.

2. Materials and methods

2.1. Reagents

Enzymes, coenzymes were supplied by Roche (Meylan, France). Williams' medium E was purchased from Gibco BRL (Cergy-Pontoise, France). L-Glutamine, valproate and dimethylsulfoxide (DMSO) were obtained from Sigma Chemicals (St Louis, MO, USA). L-[1-^{14}C] glutamine was synthesized by the method of Baverel and Lund (1979) from [1-^{14}C] glutamate (50 mCi /mmol) obtained from Amersham International (Amersham, Bucks, UK). The other chemicals used were of analytical grade.

2.2. Preparation of precision-cut renal cortical slices

Fresh human normal kidney cortex was obtained from the uninvolved pole of kidneys removed for neoplasm from 18 h fasted patients. The kidneys were decapsulated and cylindrical cores of 5 mm diameter were prepared from cortical sections with a tissue coring tool (Alabama R&D, AL, USA). The cores were maintained in oxygenated (95%O_2–5%CO_2) ice-cold Krebs–Henseleit buffer and transferred to a Krumdieck tissue slicer (Alabama R&D, AL, USA) filled with oxygenated ice-cold Krebs–Henseleit buffer. Preliminary experiments were used to determine settings that produced slices of approximately 250 μm thickness. These slices were kept in ice-cold oxygenated Krebs–Henseleit buffer until incubation or cryopreservation.

2.3. Slices cryopreservation

Prior to freezing, the slices were placed for 30 min with gentle orbital shaking in ice-cold 12% (v/v) dimethylsulfoxide (DMSO) in Williams' medium E (2 ml/slice) as described by Vanhulle et al. (2001); then, they were rapidly frozen in liquid nitrogen as described by de Kanter et al. (1998). The frozen slices were stored in cryotubes in liquid nitrogen vapor until use. For experiments, the cryopreserved slices were thawed for five minutes by direct immersion into a bath containing oxygenated Krebs–Henseleit medium at 37 °C (2 ml medium/slice); then, they were washed twice with the same medium at ambient temperature to remove DMSO.

2.4. Incubation procedure

Incubations were performed at 37 °C in Krebs–Henseleit medium in a shaking water bath in 25 ml stoppered

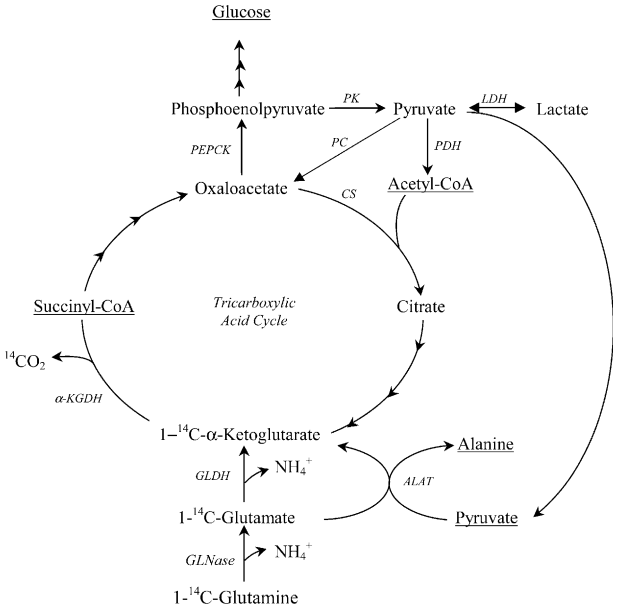

Fig. 1. Schematic representation of glutamine and lactate metabolism in human precision-cut renal cortical slices. This scheme shows that (i) the C-1 of [1-^{14}C]glutamine is released as $^{14}CO_2$ by the α-ketoglutarate dehydrogenase reaction and (ii) [1-^{14}C]α-ketoglutarate is formed by the glutamate dehydrogenase and the alanine aminotransferase reactions. GLNase, glutaminase; GLDH, glutamate dehydrogenase; ALAT, alanine aminotransferase; α-KGDH, α-ketoglutarate dehydrogenase; PEPCK, phosphoenolpyruvate carboxykinase; PK, pyruvate kinase; LDH, lactate dehydrogenase; CS, citrate synthase; PC, pyruvate carboxylase.

Erlenmeyer flasks with an atmosphere of 95%O_2–5%CO_2. Four slices were incubated in 2 ml Krebs–Henseleit buffer (pH 7.4) with 2 mM glutamine as substrate in the absence or the presence of 1 mM valproate. Incubations were terminated by adding perchloric acid [2% (v/v), final concentration]. In flasks in which lactate dehydrogenase (LDH) activity was measured (in two flasks per experimental condition in each experiment), one slice was used for intracellular LDH activity measurement and an aliquot of the medium was collected for extracellular LDH activity measurement before perchloric acid addition. In each experiment, zero time flasks were prepared with slices by adding perchloric acid before the slices. In all experiments, each experimental condition was performed at least in duplicate. When radioactive glutamine (0.1 μCi/flask) was present in the medium, incubation, deproteinization, collection and measurement of $^{14}CO_2$ were carried out as described by Baverel and Lund (1979).

2.5. Analytical methods

2.5.1. ATP and protein content

After the incubation, two slices were homogenized in 0.2 ml of cold 7% $HClO_4$ (v/v) using an Ultraturrax homogenizer at 9500 rpm; then, after centrifugation of the homogenate for 5 min at 3000 *g*, an aliquot of the supernatant was neutralized with 20% KOH (w/v)/1% H_3PO_4 (v/v) before ATP measurement. The slice ATP concentration was quantified by using the method of Lamprecht and Trautschold (1974). Pellets were solubilized in 0.5 M NaOH for protein determination. Total protein was determined in all flasks according to the method of Lowry et al. (1951) using bovine serum albumin as standard.

2.5.2. LDH release

At the end of the incubation period, slices were removed (one slice in two independent flasks for each experimental condition studied), then frozen in liquid nitrogen and thawed three times to release intracellular LDH. Enzyme leakage was determined in the incubation medium and compared with total LDH activity (extracellular + intracellular LDH). LDH activity was determined using the method described by Bergmeyer and Bernt (1974).

2.5.3. Metabolite assays

After removal of the denaturated protein by centrifugation (3000 *g* for 5 min), the supernatant was neutralized with 20% KOH/1% H_3PO_4. Glutamine, glutamate, ammonia, alanine, glucose and lactate were determined in all flasks as previously described (Baverel and Lund, 1979; Baverel et al., 1978).

2.6. Calculations and statistical analysis

Net substrate utilization and product formation were calculated as the difference between the total flasks contents at the start (zero time flasks) and after the period of incubation. $^{14}CO_2$ production was calculated by dividing the radioactivity in $^{14}CO_2$ by the specific radioactivity of the labeled glutamine determined in zero-time samples.

Data, presented as means ± S.E.M., are expressed in μmoles of metabolite produced or removed per gram of protein per incubation time (2 or 4 h). Comparisons of two groups were made by the Student's *t*-test for paired data. Statistical differences in glutamine metabolism parameters were analysed by two-way ANOVA with the interaction cryopreservation/valproate treatment followed by the Scheffe's test.

3. Results

3.1. Effect of cryopreservation on cellular viability of human precision-cut renal cortical slices

In each experiment, the effect of cryopreservation was studied in cryopreserved and then thawed (hereafter referred to as cryopreserved) human renal cortical slices incubated for 2 or 4 h in Krebs–Henseleit medium and compared with fresh slices incubated under the same conditions immediately after preparation. The slices were thawed up to 2 weeks after rapid freezing and the duration of cryopreservation did not influence the results obtained. The ATP level was measured as an index of cellular energy metabolism whereas LDH release into the incubation medium was taken as a marker of cell lysis. Moreover, the capacity of slices to maintain their metabolic activity after 4 h of incubation was studied by measuring glutamine removal and ammonia accumulation as a function of time. Fig. 2(a and b) shows that, although they were not perfectly linear with time, glutamine removal and ammonia accumulation increased with time in both cryopreserved and fresh slices. Interestingly, the glutamine removal and the accumulation of ammonia observed in cryopreserved slices were not smaller than those in fresh slices.

As shown in Table 1, the slice ATP levels were decreased in cryopreserved slices; indeed, they represented 75% and 58% of those measured in fresh slices after 2 and 4 h of incubation, respectively. After 2 h of incubation, the protein content in cryopreserved slices was not statistically different from that in fresh slices whereas it significantly decreased by 20% in cryopreserved when compared with fresh slices after 4 h of incubation. The LDH release was less than 0.5% of the intracellular LDH both in fresh and cryopreserved slices after 2 and 4 h of incubation; at zero-time, after 2 and 4 h of incubation, the intracellular LDH activities were

701±28, 682±73, and 680±88 μmoles/g protein/min in fresh slices, and extracellular LDH activities after 2 and 4 h of incubation were 4±1 and 2±1 μmol/g protein/min, respectively. In cryopreserved slices, the corresponding values were 517±40, 528±72, 547±69, 1±0.2, and 0.4±0.1 μmol/g protein/min, respectively.

3.2. Effect of cryopreservation and valproate on glutamine metabolism in human precision-cut renal cortical slices

Table 2 shows the results of experiments in which the metabolism of glutamine was studied in fresh and in cryopreserved slices both in the absence and the presence of valproate.

3.2.1. Effect of cryopreservation

It can be seen in Table 2 that, in the absence of valproate, cryopreservation did not significantly alter glutamine removal and ammonia, alanine and lactate productions by the slices. By contrast, cryopreserved slices accumulated more glutamate than fresh slices. Negligible amounts of glucose (mean values = 11 and 6 μmol/g protein/4 h), were formed in fresh and cryopreserved slices, respectively.

The release of $^{14}CO_2$ from 2 mM [1-^{14}C] glutamine, which occurs at the α-ketoglutarate dehydrogenase reaction level, was significantly reduced after 4 h of incubation in cryopreserved slices when compared with fresh slices (158.2±24.6 and 218.6±12.4 μmol/g protein/4 h, respectively; $n = 4$ experiments; $P < 0.05$; see Fig. 1 for the pathways and the fates of the C-1 of glutamine). In the absence of change in alanine production caused by cryopreservation (Table 2), this indicates a decrease in flux through glutamate dehydrogenase because no α-ketoglutarate accumulated.

3.2.2. Effect of valproate

The effect of valproate, a compound known to increase glutamine removal and ammonia production in isolated human kidney tubules (Martin et al., 1990), was

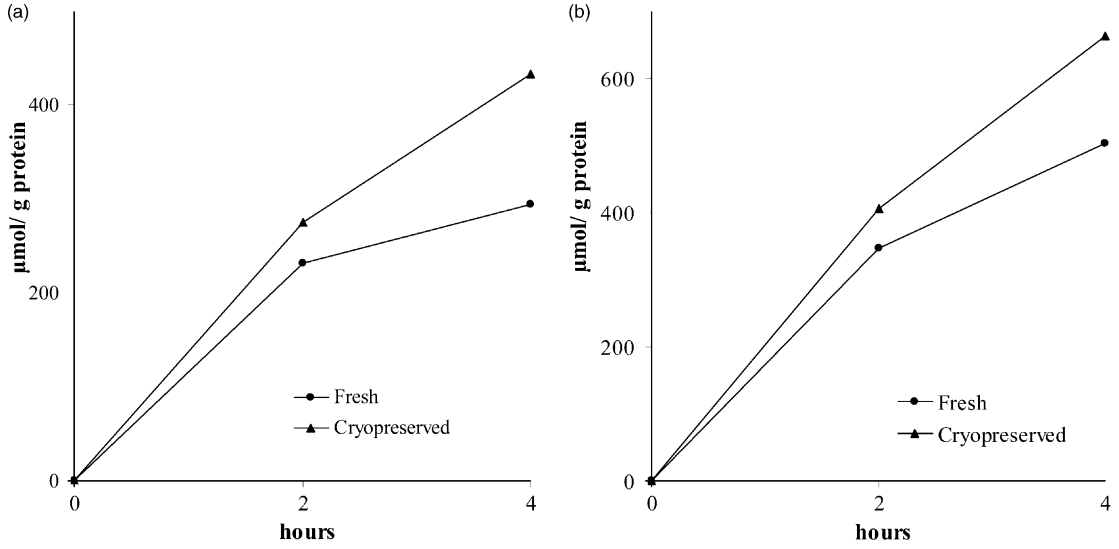

Fig. 2. Time course of glutamine removal (a) and ammonia production (b) in fresh and cryopreserved human precision-cut renal cortical slices. The values are means of two experiments performed in triplicate.

Table 1
Protein and ATP contents of human precision-cut renal cortical slices

Incubation time	Experimental condition	Protein content (mg)	ATP content (μmol/g protein)
2 h	Fresh slices	3.9±0.2	6.9±0.4
	Cryopreserved slices	3.3±0.3	5.2±0.4[a]
4 h	Fresh slices	4.0±0.2	6.9±0.8
	Cryopreserved slices	3.2±0.6[a]	4.0±0.7[a]

Fresh and cryopreserved renal cortical slices were incubated for 2 and 4 h in Krebs–Henseleit buffer in the presence of 2 mM glutamine as substrate. Results are expressed as means±S.E.M. for four experiments. Statistical difference was analyzed by the Student's *t*-test for paired data, comparing cryopreserved and fresh slices incubated for the same time.

[a] $P < 0.05$.

Table 2
Effect of cryopreservation and valproate on glutamine metabolism in human precision-cut renal cortical slices

Experimental condition	Protein content (mg)	Metabolite removal (−) or production (μmol/ g protein/4 h)				
		Glutamine	Glutamate	Ammonia	Alanine	Lactate
Fresh slices						
Glutamine (2 mM)	1.9±0.2	−438.5±39.3	281.7±36.5	663.3±55.7	71.8±9.8	35.0±5.4
Glutamine (2 mM) + Valproate (1 mM)	2.1±0.2	−625.1±30.0[a]	278.2±25.1	921.2±44.7[a]	170.6±14.0[a]	77.6±11.7[a]
Cryopreserved slices						
Glutamine (2 mM)	1.9±0.1	−493.2±41.5	412.0±47.2[b]	728.6±30.8	75.3±9.7	50.8±7.0
Glutamine (2 mM) + Valproate (1 mM)	1.8±0.1	−642.6±43.7[c]	394.1±39.4[d]	964.2±33.2[c]	129.2±20.1[c]	91.7±12.7[c]

Fresh and cryopreserved renal cortical slices were incubated for 4 h in Krebs–Henseleit buffer, in the presence of 2 mM glutamine and in absence or presence of 1 mM valproate. Net substrate utilization and product formation were calculated as the difference between the incubated flasks and the zero-time flask. Results are expressed in μmol substance removed or produced per g protein per 4 h. Values are means±S.E.M. for five experiments. Statistical difference was analyzed by ANOVA followed by the Scheffe's test.

[a] Statistically significant ($P<0.05$) fresh slices compared to fresh slices + valproate.

[b] Statistically significant ($P<0.05$) cryopreserved slices compared to fresh slices.

[c] Statistically significant ($P<0.05$) cryopreserved slices compared to cryopreserved slices + valproate.

Table 3
Effect of cryopreservation on lactate metabolism in human precision-cut renal cortical slices

Experimental condition	Protein content (mg)	Metabolite removal (−) or production (μmol/g protein/4 h)			
		Lactate	Pyruvate	Alanine	Glucose
Fresh slices	4.3±0.3	−376.8±30.4	32.4±2.0	63.2±2.4	69.9±7.0
Cryopreserved slices	3.7±0.2[a]	−336.5±23.4	36.1±2.0	79.0±3.6[a]	40.8±4.6[a]

Fresh and cryopreserved renal cortical slices were incubated for 4 h in Krebs–Henseleit buffer in the presence of 2 mM L-lactate. Net substrate utilization and product formation were calculated as the difference between the incubated flasks and the zero-time flask. Results are expressed in μmol of substance removed or produced per g protein per 4 h. Values are means±S.E.M. for 10 experiments. Statistical difference was analyzed by the paired Student's *t*-test.

[a] $P<0.05$.

studied in fresh slices incubated for 4 h with 2 mM glutamine. The concentration of valproate chosen (1 mM) was found to produce the largest effects in isolated human kidney tubules (Martin et al., 1990). As shown in Table 2, valproate addition caused large increases in glutamine utilization and in ammonia, alanine and lactate productions that were similar in fresh and cryopreserved slices. Both in fresh and cryopreserved slices, valproate did not alter glutamate accumulation.

3.3. Effect of cryopreservation on lactate metabolism in human precision-cut renal cortical slices

In order to test if the very low rates of glucose production from glutamine were due to a loss by the fresh and cryopreserved slices of their gluconeogenic capacity, we studied the metabolism of L-lactate, a physiological substrate of the human kidney and a well-established glucose precursor in isolated human renal proximal tubules or segments (Conjard et al., 2001; Baverel et al., 1979; Dubourg et al., 2001). The data presented in Table 3 show that the slices avidly used lactate as substrate and accumulated pyruvate at rates that were not statistically different in fresh and cryopreserved slices. They also show that cryopreservation led to a 25% increase in alanine accumulation and to a 42% reduction of glucose production.

4. Discussion

This study was undertaken to know if cryopreserved slices from human kidney, that have been partially characterized by other authors (Fisher et al., 1993), could be used for metabolic and pharmaco-toxicological studies related to glutamine metabolism. Among the different metabolisms present in the renal cortex, we have chosen to study glutamine metabolism whose pathways presented in Fig. 1 are important not only for

the defense of the systemic acid-base balance (Curthoys and Watford, 1995; Nissim, 1999; Welbourne et al., 2001) but also for the provision of energy for the renal reabsorptive work (Halperin et al., 1982).

4.1. Metabolic viability of fresh human precision-cut renal cortical slices

Our study demonstrates that fresh precision-cut human renal cortical slices remain metabolically viable for at least 4 h of incubation in the presence of 2 mM glutamine as substrate. However, as expected because of the diminished contact surface of the cells with the incubation medium and substrate, it can be calculated on the same time basis (1 h) that these slices used about two times less glutamine (2 mM) than isolated tubules incubated with 1 mM glutamine as substrate (Martin et al., 1990). The fact that, after 2 and 4 h of incubation, the production of ammonia exceeded by far the removal of glutamine (Fig. 2 and Table 2) indicates that not only the glutaminase reaction, which releases as ammonia the glutamine amide nitrogen, but also the oxygen-dependent glutamate dehydrogenase reaction, which releases as ammonia the amino nitrogen of glutamine, were well functional in our slices.

It is of interest to note that, unlike isolated human proximal tubules (Dubourg et al., 2001), our slices did not release LDH. This clearly indicates that the integrity of the plasma membrane of their cells that were not exposed to collagenase was well preserved. It should also be emphasized that the slice ATP content (Table 1), a marker of cellular energy metabolism, was not far from that measured in isolated human proximal tubules (Dubourg et al., 2001). Furthermore, the production of significant amounts of both lactate and alanine by the lactate dehydrogenase and alanine aminotransferase reactions, respectively, is indicative that the pathway from α-ketoglutarate to pyruvate also operated satisfactorily (see Fig. 1).

That negligible amounts of glucose were formed from glutamine may appear at first sight surprising because isotopic studies performed in vivo (Meyer et al., 1998) and measurement of the production of glucose by isolated proximal tubules (Martin et al., 1990) and microdissected proximal segments (Conjard et al., 2001) suggest that glucose is a product of human renal glutamine metabolism. In fact, when corrected for the glucose formed from endogenous substrates by isolated proximal tubules (Martin et al., 1990), or for the glucose present at the start of incubation in microdissected human proximal segments (Conjard et al., 2001), glutamine might appear to be a poor gluconeogenic precursor in human proximal tubular cells in vitro. In agreement with this view is the observation that, like in isolated human renal proximal tubules or segments (Conjard et al., 2002; Baverel et al., 1979; Dubourg et al., 2001), glucose was formed at high rates from lactate in our fresh human renal cortical slices (see Table 3 and Fig. 1).

4.2. Metabolic viability of cryopreserved human precision-cut renal cortical slices

To our surprise, our cryopreserved human renal cortical slices retained their full capacity to remove glutamine; this indicates that, in contrast with the reported instability of human renal glutaminase when exposed to cold (Mattenheimer and DeBruin, 1970), this enzyme remained stable and perfectly functional in human renal cortical slices under our cryopreservation and thawing conditions. A deleterious effect of cryopreservation on glutamine metabolism appears to be an increased accumulation of glutamate when compared with fresh slices (Table 2). Since this increased glutamate accumulation was not due to a statistically significant stimulation of glutamine removal nor to a diminished alanine synthesis (see Table 2 and Fig. 1), it probably resulted from a diminished flux through glutamate dehydrogenase, an oxygen-dependent reaction. A reduced operation of the latter enzyme would be consistent with an impairment of oxygen-dependent reactions. In agreement with this explanation, is (i) the observation that cryopreserved slices show a partial uncoupling of oxidative phosphorylation (Fuller et al., 1989), (ii) the reduced cellular level of ATP observed in our cryopreserved slices (Table 1), (iii) the reduced glucose synthesis from lactate, an ATP-dependent process (see Table 3), and (iv) that the release of $^{14}CO_2$ from 2 mM [1-^{14}C] glutamine was significantly reduced after 4 h of incubation in cryopreserved slices when compared with fresh slices (see Section 3). However, such an explanation is not consistent with the absence of a decrease in ammonia production, probably because the decrease in the production of ammonia by glutamate dehydrogenase was masked by the concurrent increase in ammonia production resulting from the increases (that were not statistically significant, see Table 2) in glutamine removal and ammonia release by glutaminase.

Thus, the mitochondria were clearly the target of the deleterious effects of our cryopreservation procedure. Whether this was due to a more pronounced growth of ice crystals in the mitochondria during freezing (then leading to some uncoupling of oxidative phosphorylation after thawing) or to "a reoxygenation damage" due to the use of an oxygenated Krebs–Henseleit buffer remains to be determined.

4.3. Reactivity to the pharmaco-toxicological effects of valproate

The data presented in Table 2 clearly demonstrate that, like in isolated human proximal tubules (Martin et

al., 1990), valproate stimulated glutamine removal and metabolism in fresh human kidney slices. Moreover, the data shown in Table 3 reveal that the ammoniagenic activity of valproate was fully preserved in cryopreserved slices. Like in kidney tubules (Martin et al., 1990), valproate stimulated not only flux through glutaminase (taken as the removal of glutamine) but also flux through glutamate dehydrogenase in slices. Indeed, the increase in ammonia production caused by valproate was greater than the increase in glutamine removal (Table 2) which necessarily means that valproate stimulated flux through the latter enzyme. Like in fresh slices, valproate did not alter glutamate accumulation but stimulated both lactate and alanine production in cryopreserved slices.

In conclusion, our study establishes that properly cryopreserved and thawed precision-cut human renal cortical slices remain metabolically viable as far as glutamine and lactate metabolisms are concerned. A deleterious effect of cryopreservation seems to be a reduction of the mitochondrial oxidative metabolism leading to a decrease in the cellular ATP levels, in lactate gluconeogenesis and in flux through glutamate dehydrogenase. Our data also demonstrate that these cryopreserved slices fully retain their capacity to respond to the stimulatory effect of valproate on ammoniagenesis from glutamine. These results show that, when obtained in large amounts, human renal cortical tissue can be stored in liquid nitrogen and used later for metabolic and pharmaco-toxicological studies.

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