Protective Effect of Fructose on Survival and Metabolic Capacities of Hepatocytes Kept Overnight under Cold Hypoxia before Normothermic Reoxygenation

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Abstract—The protective effect of fructose with regard to hypoxia-induced cell injury in overnight cold preserved hepatocytes (20 hr at 4°C) was investigated. The addition of fructose (at 10 and 20 mM) resulted in an improved survival of hepatocytes during their normothermic (37°C) reoxygenation, irrespective of the time of fructose addition before the onset of hypoxia (i.e. 10, 20 or 30 min). Such a protective effect was even higher than that observed when hepatocytes were incubated in the University of Wisconsin solution (UW). Moreover, neither Desferal (an iron chelator) nor adenosine (an ATP precursor), nor other carbohydrates (glucose, galactose and the antioxidant mannitol) were able to protect cells against such an hypoxia-mediated injury. The intracellular ATP content was lower in both adenosine- and fructose-treated hepatocytes than in control untreated cells. However, the cellular metabolic capacities such as protein synthesis and gluconeogenesis from lactate recovered faster during reoxygenation of previously hypoxic fructose-treated cells compared with both control and adenosine-treated cells. © 1997 Published by Elsevier Science Ltd

Abbreviations: BSA = bovine serum albumin; LDH = lactate dehydrogenase; PCA = perchloric acid; UW = University of Wisconsin.

INTRODUCTION

The preservation of liver during hypoxic storage and transportation is of prime importance for successful liver transplantation. At present, simple cold storage of liver in artificial medium such as the University of Wisconsin solution (UW) is used, but preservation time is limited (Todo et al., 1989). Interestingly, despite the large body of evidence showing that fructose protects hepatocytes against hypoxic injury (Anundi et al., 1987; Buc Calderon et al., 1993; Gasbarrini et al., 1992; Okabe et al., 1991), such a preservative solution (as well as other commercial media) does not contain this excellent glycolytic substrate. Indeed, since mitochondrial oxidative phosphorylation will be stopped under hypoxia, it seems nonsense to deplete ATP (due to phosphorylation of fructose to yield fructose 1-phosphate) just before the onset of the hypoxia.

All previous studies, however, have been performed under normothermic hypoxia followed, in some cases, by normothermic reoxygenation. Such an experimental condition is far from the human liver transplantation procedures. Thus, in order to mimic such conditions as closely as possible, the aim of this work was to evaluate the effect of carbohydrates (as potential glycolytic substrates) and adenosine (as an ATP precursor) on rat hepatocytes first stored under hypoxic cold conditions (20 hr at 4°C) and then subjected to normothermic (37°C) reoxygenation. Indeed, the development of strategies that would improve the survival as well as the maintenance of the metabolic capacities of the preserved organ is of clinical importance. Therefore, cell survival was assessed by incubating hepatocytes in the UW solution as well as in the presence of different carbohydrates such as fructose, glucose, galactose and the antioxidant mannitol, and of Desferal, an iron chelator with cellular protective effects during normothermic hypoxia (Khan and O’Brien, 1995; Lefebvre and Buc Calderon, 1995). The metabolic capacities of hepatocytes under such hypoxic/reoxygenation conditions were evaluated by measuring the cellular ability to form glucose from lactate (gluconeogenesis) and to synthesize proteins (as estimated by the incorporation of radiolabelled leucine into proteins), in the absence of any sub-
strate or in the presence of fructose or adenosine, an ATP precursor commonly used in cold liver preservative solutions (Southard et al., 1990).

MATERIALS AND METHODS

Animals

Male Wistar rats weighing 250–300 g were purchased from Iffa-Credo (Les Oncins-France) and housed in individual cages in a temperature- and light-controlled room (12-hr dark/light cycle). They received standard diet A03 (U.A.R., Villemoisson-sur-Orge, France) and water ad lib. Animals were fasted overnight before the isolation of hepatocytes.

Chemicals

Bovine serum albumin (BSA, fraction V), adenosine and mannitol were purchased from Sigma Chemical Co. (St Louis, MO, USA). L-[U-14C]Leucine was purchased from the Radiochemical Centre (Amersham, UK). Collagenase A (0.269 U/mg) was obtained from Boehringer Mannheim (Germany). Desferal (desferrioxamine mesylate) was purchased from Ciba-Geigy (Switzerland). Glucose, galactose and fructose were from Merck (Darmstadt, Germany). ViaSpan (Belzer University of Wisconsin solution) was purchased from Therabel (Belgium). All other chemicals and reagents were of the purest grade available.

Preparation and incubation of isolated hepatocytes

Liver perfusion was carried out under pentobarbital (60 mg/kg) anaesthesia. Hepatocytes were isolated according to the standard procedure described by Berry and Friend (1969) and slightly modified by Krack et al. (1980). Isolated cells were suspended in 40 ml Krebs–Henseleit glucose-free medium (NaCl 120 mM; KCl 5 mM KH2PO4 1 mM; MgSO4 1 mM; CaCl2 2.5 mM; NaHCO3 25 mM; pH 7.4), containing 0.3% BSA, at a final concentration of 2 × 10⁶ cells/ml.

The experimental protocol (Fig. 1) was as follows: cells were incubated in a shaking water-bath at 37°C for 60 min under a continuous flowing atmosphere of O2/CO2, 95%/5% (aerobic conditions). Afterwards, cells were placed for 10 min under a flow of N2/CO2, 95%/5% (anaerobic conditions) to purge oxygen and to induce an hypoxic process, and they were kept at 4°C without shaking for 20 hr. After such hypoxic cold preservation conditions, hepatocytes were reoxygenated (O2/CO2, 95%/5%) and further incubated in a shaking water-bath at 37°C for 120 min (or 180 min in the case of protein synthesis and gluconeogenesis measurements). The addition of compounds was performed as indicated in the figures legends.

Viability test

Viability of hepatocytes was estimated by measuring the activity of lactate dehydrogenase (LDH) according to the procedure of Wroblewski and Ladue (1955), both in the culture medium and in the cell pellet obtained after the centrifugation as described previously (Krack et al., 1980). LDH leakage was expressed as a percentage of the combined total.

Biochemical assays

Cellular ATP was measured on neutralized perchloric acid extracts as described elsewhere (Lefebvre et al., 1993) by using an HPLC-LKB

![Diagram](image-url)

Fig. 1. Experimental protocol for incubation of hepatocytes exposed first to cold hypoxia (4°C) and then to normothermic (37°C) reoxygenation. A = time before hypoxia (min); B = time before hypothermia (min); C = time of cold hypoxia (20 hr); D = time of normothermic reoxygenation (min). The different carbohydrates (fructose, glucose, galactose and mannitol) were added 10 min before the onset of hypoxia (−10 in A). In some experiments fructose was added 30, 20 or 10 min before the onset of hypoxia (−30, −20 or −10 in A). Desferal was added at the beginning of the incubation (−60 in A), whereas adenosine was added 10 min after the induction of hypoxia but before the hypothermia (+10 in B). For incubations with UW solution, hepatocytes were incubated in UW from the outset (−60 in A), but at 4°C instead of 37°C. Radiolabelled leucine for protein synthesis and lactate/pyruvate for gluconeogenesis were added at the end of cold hypoxia (C) before the reoxygenation at 37°C (D). Sampling times were (1) at −60 min, (2) at +10 min of hypoxia, (3) at 0 min of reoxygenation and (4) at 120 min of reoxygenation.
Pharmacia instrument equipped with a 4.7 x 125 mm (particle size 5 μm) anion exchange column (Partisphere SAX, Whatman). Separation was achieved by the use of an isocratic elution mode with 0.45 M NH₄H₂PO₄, pH 3.7 at a flow rate of 1.5 ml/min. The results are expressed as nmol/mg protein.

Protein synthesis was estimated by measuring the incorporation of [14C]leucine (sp. act. 94 μCi/mmol; 0.8 mM unlabelled leucine) into the protein pellet following precipitation with perchloric acid (HClO₄; PCA) as reported by Seglen (1976). Results are expressed as dpm/mg protein in a PCA precipitate. The amount of protein was determined by the method of Lowry et al. (1951) using BSA as standard.

Gluconeogenesis from lactate (glucose formation) was measured with the glucose oxidase method, as described elsewhere (Hue et al., 1975), and results are expressed as nmol of glucose formed/mg protein.

Statistics

The results are expressed as mean values ± standard error of the mean (SEM) of at least three separate experiments. Analysis of variance (two-way ANOVA with the interaction time/treatment) was used to compare the respective parameters. For statistical comparison of results at a given time point, data were analysed using Student’s t-test. The level of significance was set at P < 0.05.

RESULTS

Figure 2 shows the time course of LDH leakage of hepatocytes exposed to the transition cold hypoxia/normothermic reoxygenation procedure. Comparison of LDH leakage was made between hepatocytes incubated in the absence of any glycolytic substrate (control untreated cells) or in the presence of either carbohydrates (20 mM), or Desferal (5 mM) when added either 10 or 60 min before the onset of hypoxia, respectively. The addition of fructose resulted in a better protection against cellular injury than in either untreated cells or those given other treatments. The effect of fructose was mainly seen during the normothermic reoxygenation, whereas after 20 hr of cold hypoxia all conditions resulted in LDH leakage of around 40-60%. Compared with incubation of hepatocytes in UW solution, the preincubation with fructose resulted in the best protection against hypoxia/reoxygenation injury. Interestingly, the addition of fructose to UW solution resulted in an enhanced protection. Indeed, after 120 min of normothermic reoxygenation, the percentage LDH leakage was only 27.1 ± 3.3 (data not shown). Moreover, the normothermic reoxygenation of previously hypoxic hepatocytes first
incubated in UW solution and then washed and replaced in Krebs medium, just before reoxygenation, gave essentially the same results as unwashed cells (data not shown).

Protection by fructose was optimal for the two highest concentrations (i.e., 10 and 20 mM), giving an LDH of 40% leakage after 2 hr normothermic reoxygenation. For fructose-treated cells at 2 and 5 mM and control untreated cells, LDH leakage was 58, 61, and 67%, respectively (data not shown). Moreover, different times of fructose addition (i.e., 10, 20 or 30 min before the onset of hypoxia) resulted in similar LDH leakage of about 40% (data not shown).

Figure 3 shows the time course of the intracellular ATP content of either control cells or those treated with fructose (10 mM) or adenosine (0.5 mM). The addition of fructose, 10 min before the onset of hypoxia, decreased the intracellular ATP content from 8.6 to 3.8 nmol/mg protein. This low ATP level remained fairly constant when cells were kept under hypoxic cold conditions and further incubated at 37°C under aerobic conditions. At any time during the experimental protocol, the ATP content of fructose-treated cells was lower than that of control untreated cells. Indeed, after 20 hr cold hypoxia the intracellular content of ATP was 5.4, 3.3 and 2.9 nmol/mg protein for control, adenosine- and fructose-treated cells, respectively.

Furthermore, after 120 min of normothermic reoxygenation, these values were 4.2, 2.9 and 2.3 nmol/mg protein, respectively. However, LDH leakage was 40% in fructose-treated hepatocytes compared with 65% in adenosine-treated cells and 67% in control untreated cells (data for adenosine-treated cells not shown).

Figure 4 shows the time course of radiolabelled leucine incorporation into proteins within hepatocytes incubated for 180 min at 37°C under aerobic conditions, after 20 hr of hypoxic cold preservation. When fructose (10 mM) or adenosine (0.5 mM) pretreated cells were reoxygenated at 37°C, they started synthesizing protein again. The rate of that biosynthetic process was, however, faster in hepatocytes preincubated with fructose than with adenosine: 3.8 and 0.9 dpm of [14C]leucine incorporated/mg protein/min, respectively. The rate of protein synthesis in fructose-treated hepatocytes was about 10-fold that of control untreated cells (0.35 dpm of [14C]leucine incorporated/mg protein/min).

Under the same experimental conditions as described for the incorporation of radiolabelled leucine into proteins, the ability of cells to form glucose from lactate/pyruvate (gluconeogenesis) was also tested. Figure 5 shows that the rate of gluconeogenesis from lactate was higher in fructose-treated cells (16.8 nmol of glucose produced/mg protein/min) than in adenosine-treated cells.
(5.6 nmol of glucose produced/mg protein/min) or in control untreated cells (4.9 nmol of glucose produced/mg protein/min). When fructose-treated hepatocytes were incubated under aerobic conditions at 37°C but in the absence of lactate, the formation of glucose (most probably from fructose-1-phosphate) increased linearly during the first 90 min; it then reached a plateau and after 100 min it decreased (data not shown).

DISCUSSION

The metabolism of cells is dramatically modified during oxygen deprivation (Bontemps et al., 1993; Bronk and Gores, 1993; Kehrer et al., 1990; Seglen, 1974), but they may survive by reorganizing their metabolism. Such an adaptive response involves the shutting off of several metabolic pathways that are not essential for the survival of cells themselves but which are, however, highly energy-consuming (such as for protein synthesis or lipoprotein secretion)—the so-called metabolic arrest (Hochachka, 1986). This strategy allows cells to survive in an environment of low oxygen saturation, provided that they contain sufficient glycogen (or glycolytic substrates) to maintain ATP production by anaerobic glycolysis. Therefore, if hypoxia is long lasting and/or in the absence of substrates to support anaerobic glycolysis, the biochemical and metabolic dysfunctions lead to cell death. Conversely, if the ATP level is maintained by more efficient metabolic pathways, or by a reduction of ATP turnover, cell survival will be improved.

We have compared the effects of several compounds—involved directly or indirectly in glycolytic ATP production or with antioxidant capacity—on the capacity of cells to survive under hypoxia/reoxygenation. Of these, only fructose and UW solution were able to influence the survival of hepatocytes kept under cold hypoxia and subsequently normothermically reoxygenated. As previously suggested (Buc Calderon et al., 1993; Lefebvre et al., 1994 and 1995), we hypothesize that cells can survive when receiving fructose, because they accumulate fructose-1-phosphate during the aerobic normothermic incubation. Meanwhile, the ATP required for fructose phosphorylation can be regenerated through oxidative phosphorylation. Such a protection was not expressed during cold hypoxia because the glycolytic ATP production was not realized. Nevertheless, since metabolic requirements decrease when hypoxia occurs (metabolic arrest) and because, during normothermic reoxygenation, fructose-1-phosphate enters the glycolytic pathway at the level of triose phosphates, thus allowing a high degree of glycolytic activity (and hence a continuous ATP supply), cells remain viable much longer than hepatocytes receiving other carbohydrates, which appear to be unable to supply a glycolytic reservoir. In hepatocytes cold stored in UW solution, LDH leakage was significantly lower than in glucose-, galactose-, mannitol- or Desferal-treated cells; nevertheless after normothermic reoxygenation, this protection was less effective that that obtained with fructose (Fig. 2).

The formation of deleterious reactive oxygen intermediates has been reported during normothermic hypoxia/reoxygenation (Kehrer et al., 1990). Moreover, we and others (Khan and O’Brien, 1995; Lefebvre and Buc Calderon, 1995) have proposed that Desferal might play a protective role during hypoxia by chelating ionic iron thus avoiding its catalytic role in a Fenton reaction. Nevertheless, neither the iron chelator Desferal, nor the hydroxyl free radical scavengers mannitol and glucose (Buc Calderon and Roberfroid, 1988) were able to influence cell survival under such a protocol of hypoxia/reoxygenation. Therefore, it appears unlikely that the enhanced cell death (observed after cold hypoxia followed by normothermic reoxygenation), can be related to overproduction of free radicals during the reoxygenation step. It is most likely that such a cytotoxicity results from metabolic effects associated with the change of temperature from 4°C to 37°C.

There is increasing clinical evidence to suggest that successful liver transplantation is more closely related to the metabolic competence of the transplanted organ (e.g. gluconeogenic capacity) than to its ATP content (Asonuma et al., 1991; D’Alessandro et al., 1986; Sumimoto et al., 1988). In agreement with such hypothesis, our results

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**Fig. 5.** Effect of fructose and adenosine on gluconeogenesis from lactate. Experimental conditions were described in the legend to Fig. 3. Lactate (10 mM) and pyruvate (1 mM) were added at the beginning of the normothermic reoxygenation. At the times indicated, aliquots of cell suspension were taken and the glucose formation was measured as described in Materials and Methods. Results are expressed as nmol glucose/mg protein and they represent mean values ± SEM of three separate determinations. ANOVA test: two-factor interactions (time/treatment) significance level P < 0.001 for the comparison between fructose-treated hepatocytes and both adenosine-treated and control untreated hepatocytes.
show that, despite a low ATP content, cell survival was best in fructose-treated cells. Moreover, with regard to both metabolic abilities (i.e. protein synthesis and gluconeogenesis from lactate) during normothermic reoxygenation, the results reported herein clearly show a faster recovery of both capacities in hepatocytes previously incubated in the presence of fructose compared with that in the presence of adenosine. It must be underlined that the rate of gluconeogenesis is very similar to that reported by us for fructose-treated cells freshly incubated for 60 min under aerobic conditions at 37°C (about 20 nmol of glucose produced/mg protein/min) (Lefebvre et al., 1994). Since liver acceptors are under conditions of lactic acidosis, such a gluconeogenic activity from lactate represents an interesting feature of fructose pretreatment. Nevertheless, the rate of protein synthesis remains low compared with that which we observed in hepatocytes also receiving fructose but freshly incubated at 37°C under aerobic conditions (about 10 dpm of [14C]leucine incorporated/mg protein/min) and after 90 min normothermic hypoxia (Lefebvre et al., 1994).

The lack of effect of adenosine on protein synthesis is not unexpected. Indeed, we have reported that, in aerobic conditions, extracellular adenosine inhibits an inhibition of protein synthesis, most probably by affecting transmethylation reactions (Tinton and Buc Calderon, 1995a,b; Tinton et al., 1995). Moreover, a large amount of extracellular adenosine, such as is commonly used in organ-preservation solutions (Southard et al., 1990), might induce many physiological or unphysiological responses in the presence of P1 purinoceptors (Burnstock, 1993) and, therefore, it may be at the root of many cardiac and haemodynamic disturbances during the reperfusion step.

In conclusion, both the accumulation of fructose-1-phosphate and the metabolic arrest during hypoxia allow a fast reconditioning of all metabolic activities during reoxygenation, which guarantees healthy functioning of the transplanted organ. Indeed, when cells are in metabolic arrest during hypoxia, the ATP requirement will be kept to a minimum and its glycolytic production from fructose-1-phosphate is used mainly for the maintenance of critical functions, such as the activity of ionic pumps. A similar approach has been used to help preserve the viability of hepatocytes isolated from fasted rats and cold stored for 24 hr, to which fructose was added as an energy-giving substrate before being rewarmed (Vreugdenhil et al., 1993).

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