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FREE RADICALS APPEAR TO AFFECT SURVIVAL OF HEPATOCYTES AT 4°C

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1) Rat hepatocytes, stored in a simple salts medium for 24 h at 4°C, retain more than 80% of their capacity to synthesize glucose from lactate.

2) The combination of NH_4Cl with oleate is cytotoxic during storage and during subsequent incubation of hepatocytes from 48 h starved rats, but not to hepatocytes from fed rats.

3) Protection against cytotoxicity is afforded by albumin and by a number of other compounds, notably polyols and glycerol.

4) These compounds appear to exert their effects by scavenging free radicals and, in the case of polyols and glycerol, by supplying reducing equivalents to maintain the redox state of the cell in the face of increased flux through glutathione peroxidase.

Key words: hepatocytes; gluconeogenesis; free radical scavengers, 4°C

INTRODUCTION

We have reported that hepatocytes maintain about 80% of their capacity to synthesize glucose and urea after storage for 24 h in a simple salts medium provided that albumin is also present[1]. Attempts were then made to improve survival of the metabolic integrity of the cells by storing them in the presence of xylitol, sorbitol or glycerol. These compounds, unlike glucose, readily enter the glycolytic pathway in hepatocytes and, by generating ATP, might be expected to provide some protection from the hypoxic conditions of storage. To our surprise, after exposure to xylitol, sorbitol or glycerol for 24 h at 4°C, hepatocytes subsequently synthesized glucose from the polyol or glycerol at a rate at least 40% higher than did the fresh suspension before storage. Moreover, the increased rate occurred even in the absence of albumin. In the previous work[1], NH_4Cl, lactate, pyruvate, oleate and ornithine had been present simultaneously for measurement of both glucose and urea synthesis. The requirement for albumin under these conditions, but not when a polyol or glycerol was the substrate, indicated that the combination of substrates must contain a cytotoxic component. This paper is concerned with the identification of this as the combination of oleate and NH_4Cl; either alone is much less damaging. We attribute the cell damage,

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that occurs even under the hypoxic conditions of storage, to production of free radicals. Hepatocytes from starved rats are vulnerable; those from fed animals are not.

MATERIALS AND METHODS

Materials

Oleic acid and urease (type VI) were obtained from Sigma. Other chemicals, enzymes and co-enzymes were from BDH, Koch-Light or Boehringer. For details of albumin, see[1].

Treatment of Tissue

Hepatocytes were isolated from livers of fed (PRM diet; E. Dixon & Sons (Ware) Ltd.) — or 48 h starved female Wistar strain rats. The procedures for cell isolation, storage and incubation were essentially as described[1]. The number of observations in the Table refers to separate hepatocyte preparations.

Determination of Metabolites

Glucose, urea, ketone bodies and lactate were determined as described previously[1]. Reduced glutathione (GSH) in the tissue was measured by the glyoxalase method of Racker[2], with modifications[3]. For this assay the cell suspension (4 ml), containing approx. 80 mg wet wt. of tissue, was quickly centrifuged (2000 × g for 30 sec) and the pellet was deproteinized and extracted with 1 ml $HClO_4$ (3% v/v) saturated with EDTA. The supernatant was neutralized with KOH immediately before the assay, without use of Universal indicator which interferes at 240 nm.

Enzyme Assays

For assays of superoxide dismutase and glutathione peroxidase, cell suspensions were treated with digitonin (approx. 0.5 mg/100 mg wet wt. hepatocytes) at 22°C for 1 h, and checked under the microscope to be certain that all cells took up trypan blue. The suspension was then sonicated at 0°C (4 × 15 sec); see[4]. Enzyme activities were measured on the supernatant (27,000 × g for 10 min). All assays were carried out at 22°C. Superoxide dismutase was assayed in the system described under "Test for Free Radical Scavengers." The amount of wet wt. of hepatocytes required to inhibit the rate of reduction of cytochrome c by 50% is defined as 1 unit of activity (see[5]). Glutathione peroxidase was determined spectrophotometrically at 340 nm[6]. The assay system was modified to contain 80 mM K phosphate buffer, pH 7.0, 1 mM diethylenetriaminepenta-acetic acid, 0.2 mM NADPH, 1 mM GSH, 1 mM t-butylhydroperoxide, 2 units glutathione reductase and tissue extract in 3 ml final volume. Activity was shown to be proportional to amount of tissue extract. Activities were corrected for non-specific oxidation of NADPH.

Test for Free Radical Scavengers

Compounds were tested for their capacity to inhibit the re-oxidation of reduced

cytochrome c in a system similar to that described by Beauchamp & Fridovich[7]. Cuvettes contained (final concentration): 0.1 M K-phosphate buffer, pH 7; 8 μM cytochrome c; 33 μM xanthine; 0.1 mM EDTA. The mixture was saturated with O_2. Reduction of cytochrome c on addition of xanthine oxidase (0.01 U/ml) was recorded at 550 nm (Aminco-Chance DW2 double beam spectrophotometer) at 0.1 full scale absorbance. Complete reduction occurred under these conditions. In the presence of 0.24 mM H_2O_2, $OH^{\cdot}$ was formed, reduction of cytochrome c by $O_2^{-\cdot}$ was almost balanced by its re-oxidation by $OH^{\cdot}$ and the reaction reached a plateau at about 0.02 on the scale. Compounds that scavenge free radicals, i.e. prevent re-oxidation of reduced cytochrome c, produce a plateau between these two extremes.

RESULTS

Cytotoxic effect of oleate in the presence of NH_4^+

The finding that albumin was not essential for survival of hepatocytes stored with polyols and glycerol made us realise that the substrate combination previously used to assess capacity of hepatocytes to synthesize urea and glucose after storage (10 mM NH_4Cl; 10 mM lactate; 1 mM pyruvate; 1 mM ornithine; 0.5 mM oleate[1]) must contain a cytotoxic component. This we have identified as the combination of oleate with NH_4Cl (Table I). When gluconeogenesis from lactate (plus lysine[8]) was used as the criterion of cell viability, survival was 90% in the presence, and 83% in the absence, of albumin. The presence of oleate or NH_4Cl alone in the absence of albumin causes the loss of about 50% biosynthetic capacity, but the two together are highly cytotoxic. Hepatocytes survive remarkably well if O_2 in the gas phase is replaced by air or N_2 (77% and 68% respectively of gluconeogenic capacity maintained; see Table I).

Trypan blue, which is generally used to assess viability of liver cell preparations, proved to be a poor indicator of the metabolic state of cell suspensions after storage. In some instances heavily stained suspensions performed well on subsequent incuba-

TABLE I
Toxicity of oleate and NH_4Cl on storage of hepatocyte suspensions at 4°C

Conditions of storage			Glucose synthesis (μmol/min per g wet wt.)		
Albumin	Substrate	Gas phase	Fresh suspension	After 24 h at 4°c	% capacity retained
2.5% w/v	None	O_2:CO_2	1.22 ± 0.10 (10)	1.10 ± 0.08 (10)	90
None	None	O_2:CO_2	0.95 ± 0.07 (4)	0.79 ± 0.05 (4)	83
None	None	Air:CO_2	0.95 ± 0.07 (4)	0.73 ± 0.10 (4)	77
None	None	N_2:CO_2	0.95 ± 0.07 (4)	0.65 ± 0.16 (4)	68
None	NH_4Cl; oleate	O_2:CO_2	0.71 ± 0.17 (3)	<0.05 (3)	<1
None	NH_4Cl; oleate	N_2:CO_2	0.71 ± 0.17 (3)	<0.05 (3)	<1
None	Oleate	O_2:CO_2	1.06 ± 0.08 (4)	0.60 ± 0.28 (4)	57
None	Oleate	N_2:CO_2	1.06 ± 0.08 (4)	0.44 ± 0.18 (4)	42
None	NH_4Cl	O_2:CO_2	0.79 ± 0.09 (3)	0.36 ± 0.10 (3)	46

Lactate (10 mM) (with L-lysine (2 mM)[8]) was the gluconeogenic precursor, with O_2:CO_2 (19:1) as gas phase for all incubations at 37°C. NH_4Cl (10 mM) and oleate (0.5 mM) were included, where appropriate, in the fresh suspension. After 24 h storage at 4°C, lactate and lysine were added to flasks before incubation at 37°C for 60 min. Rates are means ± S.E.M. of the number of observations in parentheses. All gas mixtures contained 5% CO_2.

tion; in others, apparently intact cells had lost their capacity to synthesize glucose. "Blebs" on the cell surface were common after storage at 4°C.

These experiments show that hepatocytes can be stored at 4°C, simply in Krebs-Henseleit[9] saline. However, during storage and subsequent incubation in the absence of albumin the cells become vulnerable to agents — in this case NH_4^+ plus oleate — which are not cytotoxic to the fresh suspension. However, this is a phenomenon that occurs in conjunction with starvation. The combination of NH_4^+ and oleate is not cytotoxic after storage of hepatocytes from fed rats: over 80% of capacity to synthesize urea from NH_4Cl, lactate, ornithine and oleate is maintained in the absence of albumin (results not shown) compared with 3% after starvation[1].

Evidence that free radicals are responsible for cytotoxic effects of oleate and NH_4^+

The various compounds to which hepatocytes had been exposed in the previous experiments[1] and in the experiments shown in Table I were tested for their ability to scavenge $OH^\cdot$ as described in the Materials and Methods section. Of the compounds tested (lactate (10 mM), pyruvate (1 mM), NH_4Cl (10 mM), ornithine (1 mM), oleate (0.5 mM), sorbitol (10 mM), glycerol (10 mM), xylitol (10 mM), mannitol (10 mM), glucose (10 mM), lysine (2 mM), defatted albumin (2% w/v)), all except oleate, lysine, NH_4Cl and ornithine were as effective, or more effective, than mannitol, a known scavenger of $OH^\cdot$, in preventing re-oxidation of reduced cytochrome c. These findings provide a possible explanation for the protective effects of albumin, polyols and glycerol. This suggestion is supported experimentally in that generation of $O_2^{-\cdot}$ (and H_2O_2) by xanthine oxidase in the presence of 1 mM hypoxanthine in albumin-free medium virtually abolishes gluconeogenesis from lactate in freshly isolated hepatocytes whereas glucogenesis from xylitol is hardly affected (results not shown).

Further circumstantial evidence of production of free radicals and of their cytotoxic effects on hepatocytes from 48 h starved rats is presented in Table II. While the quantitative effects vary from experiment to experiment, consistent observations are (i) NH_4^+ and oleate increase lactate, and decrease glucose, production from polyols and glycerol during storage at 4°C, (ii) it is during subsequent incubation, and

TABLE II
Cytotoxic effects of NH_4^+ and oleate on hepatocytes after storage at 4°C

Substrate	Albumin	After 1 h at 37°C	After 24 h at 4°C			After 1 h at 37°C following storage	
		GSH content	Lactate formed	Glucose formed	GSH content	Glucose formed	GSH content
None	−	0.64			0.59		0.07
None	+	0.61			0.83		0.44
Oleate; NH_4Cl	−	0.46			0.50		<0.03
Oleate; NH_4Cl	+	0.68			0.77		0.29
Xylitol	−		6.6	30.9		50.4	0.15
Xylitol; oleate; NH_4Cl	−		12.9	16.5		2.6	<0.03
Glycerol	−		20.6	3.7		36.4	0.37
Glycerol; oleate; NH_4Cl	−		28.7	0.7		5.1	0.07

Hepatocytes from 48 h starved rat. Concentrations were: oleate (0.5 mM), NH_4Cl (10 mM), xylitol and glycerol (10 mM), defatted albumin (2.5% w/v). For details see Materials and Methods. GSH content was 1.12 μmol per g in the freshly prepared hepatocytes. All values are expressed as μmol per g wet wt. tissue.

not during storage at 4°C, that NH_4^+ and oleate cause major losses of GSH (much less GSH is lost on incubation of fresh suspensions), and albumin, polyols and glycerol have protective effects; in the absence of albumin, ketone bodies formed from 0.5 mM oleate on incubation after storage appear largely as acetoacetate (data not shown). Unless albumin or these metabolizable alcohols are present, it appears that there is a loss of reducing equivalents from the cell that could be related to increased necessity for removal of peroxides through the glutathione peroxidase system (see Discussion). The non-metabolizable alcohols, mannitol or ethanol (with 0.1 mM pyrazole present to inhibit its metabolism) exerted no protective effects, nor did the presence of bovine erythrocyte Cu-Zn-superoxide dismutase (prepared by Dr. J. Bannister, Inorganic Chemistry Laboratory, University of Oxford) at 0.4 mg per flask and/or catalase (Boehringer), 1000 U per flask. The lack of effect of these enzymes is not necessarily evidence against the production of free radicals; it merely indicates that they were neither generated in the medium, nor released from the cell. This is in contrast to the experiments of others[10] in which protection was afforded by these enzymes against the cytotoxic effects of NH_4Cl and cysteine, possibly because free radical production on oxidation of cysteine to cystine occurs in the medium.

Superoxide dismutase and glutathione peroxidase activities in hepatocytes: effects of storage of suspension at 4°C

A possible explanation for the susceptibility of stored suspensions of cells from 48 h starved rats to NH_4Cl and oleate could be that activity of some protective enzyme is lost. Measurements of the activities of total superoxide dismutase and glutathione peroxidase show no significant losses after 24 h at 4°C. Superoxide dismutase activity (measured at the amount of tissue required to inhibit reduction of cytochrome c by 50%; see Materials and Methods section) decreased from 0.105 ± 0.02 to 0.117 ± 0.04 mg wet wt. after 24 h at 4°C. Activity of glutathione peroxidase decreased from 31.8 ± 3.2 in the fresh suspension to 25.4 ± 2.5 μmol/min per g wet wt. of hepatocytes (mean ± S.E.M. of 3 cell preparations) after 24 h at 4°C. However, assays of hepatocyte extracts from fed rats suggest that activities of the two enzymes are considerably lower in the fed state, although they do not decrease after storage of the suspension (superoxide dismutase 0.163 ± 0.003 and 0.143 ± 0.003 mg wet wt. in the fresh and 24 h stored suspensions respectively (means ± S.E.M. of 3 preparations); glutathione peroxidase 15.9 ± 5.2 and 24.0 ± 7.1 μmol/min per g wet wt. before and after storage at 4°C respectively (means ± S.E.M. of 3 preparations)). It is curious that activity of glutathione peroxidase appears to increase in all hepatocyte suspensions from fed animals stored at 4°C; the same phenomenon occurred in the sonicated, cell-free extract of the fresh suspension after 24 h at −20°C (21.9 ± 5.4 μmol/min per g hepatocytes).

DISCUSSION

Differences between hepatocytes from fed and 48 h starved rats in maintenance of biosynthetic processes after storage at 4°C

The experiments show that hepatocytes from 48 h starved rats survive remarkably well in a simple salts medium for 24 h at 4°C. Our failure to see this previously[1] was because 10 mM NH_4Cl and 0.5 mM oleate were components of the incubation media.

As a result, we came to the conclusion that albumin was essential for the maintenance of metabolic competence. It now appears that the combination of oleate and NH_4^+ is cytotoxic because of free radical production, and that albumin may exert a protective effect by scavenging free radicals. However, it is difficult to see why albumin should be so effective when other known free radical scavengers are not. It may be that it acts by binding oleate, thus keeping its free concentration low. But it is curious that the oleate/NH_4^+ combination is not cytotoxic to stored hepatocytes from fed rats, nor to fresh suspensions from fed or starved animals. Starvation itself, superimposed on 24 h storage at 4°C, must make the cells more vulnerable to free radical attack, possibly through losses of the normal antioxidant defence mechanisms (i.e. enzymes, vitamin E, lipid components of the plasma and intracellular membranes). Our measurements of activities of superoxide dismutase and glutathione peroxidase (enzymes important in disposal of $O_2^{-\cdot}$ and peroxides) show that activity of the latter enzyme decreases on storage of 48 h starved, but not of fed, hepatocytes. While activities per gramme of tissue are higher after starvation, it is fair to say that the liver size is halved on starvation of a rat for 48 h. At the same time the number of parenchymal cells increases from $4.13 \pm 0.58 \times 10^8$ (mean ± S.E.M. of 4 preparations) to $6.74 \pm 0.19 \times 10^8$ (7 preparations) per g dry wt. after 48 h starvation (P. Lund, unpublished measurements). Differences in activity per cell of these enzymes are unlikely to be the explanation for the different responses to storage of hepatocytes from fed and 48 h starved animals.

Polyols and glycerol as protective agents

We were surprised to find that polyols and glycerol appeared to be more rapidly converted to glucose by hepatocytes after storage at 4°C than by the fresh suspension (see Introduction). Their function as free radical scavengers could perhaps explain why 100% of glucogenic capacity of the hepatocytes could be maintained in the stored suspension, but not how glucogenic rates could be up to 50% higher than in the fresh suspension. A characteristic feature of xylitol, sorbitol and glycerol is that an NAD-linked oxidative step is involved at an early stage in their metabolism (D-xylulose reductase [EC 1.1.1.9], sorbitol dehydrogenase [EC 1.1.1.14] and glycerol-3-phosphate dehydrogenase [EC 1.1.1.8] respectively). This "extra" oxidative step upsets the balance of oxidation and reduction achieved by glyceraldehyde-phosphate dehydrogenase [EC 1.2.1.12] and lactate dehydrogenase in glycolysis, so that metabolism of these compounds would not necessarily be expected under the hypoxic conditions of storage. However, re-oxidation of NADH is not restricted to the electron transport chain and NAD-linked dehydrogenases. It is likely that the protective effects of polyols, glycerol, and even lactate, are related to their ability both to scavenge free radicals and to protect against the damaging effects of peroxides by generating reducing equivalents. Peroxides could be formed which would require a continuous supply of GSH for their disposal, so that the following sequence of reactions involving (1) a dehydrogenase, (2) transhydrogenase [EC 1.6.1.1.], (3) glutathione reductase [EC 1.6.4.2] and (4) glutathione peroxidase [EC.1.11.1.9], can be visualized:

$$\left.\begin{array}{l}\text{polyol}\\ \text{glycerol-3-phosphate}\\ \text{lactate}\end{array}\right\} + NAD^+ \longrightarrow \text{oxidized substrate} + NADH \qquad (1)$$

$$NADH + NADP^+ \longrightarrow NADPH + NAD^+ \qquad (2)$$

$$NADPH + H^+ + GSSG \longrightarrow 2GSH + NADP^+ \quad (3)$$
$$2GSH + H_2O_2 \longrightarrow GSSG + 2H_2O \quad (4)$$

Sum: Reduced substrate + H_2O_2 ⟶ oxidized substrate + H_2O

(In reaction (4), H_2O_2 may be replaced by a lipid hydroperoxide[11].) These reactions could explain why metabolizable (polyols, glycerol) but not non-metabolizable (mannitol, ethanol in the presence of pyrazole) alcohols exert protective effects.

In the absence of a supply of NADPH, GSH is oxidized and released from the cell[12]. Under conditions of hyperbaric O_2 the process is exacerbated by nutritional stress, for example, starvation and tocopherol deficiency[13]. Storage of hepatocytes at 4°C could be considered a nutritional stress against which the above reactions provide protection.

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