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An isothermal induction of Δ^9-desaturase in cultured carp hepatocytes

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Abstract

Cold exposure of carp leads to the induced activity of the hepatic Δ^9-desaturase (Schünke, M. and Wodtke, E. (1983) Biochim. Biophys. Acta 734, 70–75). We have investigated the controlled expression of this enzyme using isolated carp hepatocytes. Culture at 30°C, of cells isolated from 30°C-acclimated carp, resulted in an 8–13-fold increase in desaturase-specific activity over 4 days, whilst another enzyme of intermediary metabolism, glucose-6-phosphatase, decreased by more than 60%. This desaturase induction was associated with a loss of intracellular lipid vesicles and with increases in the levels of oleic acid of membrane phosphoglycerides and corresponding decreases in 22:6($n-3$). Supplementation of cultures with oleic acid and with polyunsaturated fatty acids did not cause any reduction in the desaturase induction. The level of immunodetectable desaturase protein increased during culture at 30°C and a desaturase mRNA was detected after 2 days of culture by Northern analysis. These results suggest that in vitro culture leads to an increased synthesis of desaturase protein by means of activated gene transcription. Significantly, transfer of cultures of 30°C-acclimated hepatocytes to 10°C resulted in a smaller induction of desaturase activity; thus cold transfer of cells in itself did not induce hepatocyte desaturase activity as does whole animal cooling. This suggests either that cold induction of desaturase activity in vivo involves systemic control or that the conditions imposed by culture prevent cold induction.

Keywords: Δ^9-Desaturase; Isothermal induction; Desaturase induction; Hepatocyte; Lipid vesicle; (Carp liver)

1. Introduction

An important and widespread response of living organisms to environmental cold is an increase in the proportion of unsaturated fatty acids of membrane phospholipids [1,2]. This response offsets the direct cold-induced ordering of their membranes, and is termed 'homeoviscous adaptation' [3] and is, perhaps, the clearest example of the homeostatic, cellular control of membrane lipid composition for structural, adaptive reasons. Its basis lies in the regulated control of lipid biosynthetic enzymes. Although several enzymes have been implicated in this process in eukaryotes, the mechanisms by which their activity is regulated in response to cold is not understood. In vertebrates there is one clear-cut example of induced expression which appears to be closely and perhaps causally linked with cold-induced adaptive shifts in lipid composition and membrane structural order, namely the hepatic Δ^9-desaturase of carp [4]. Enzymatic activity of this enzyme was increased following transfer of warm-acclimated carp to the cold with a time-course that corresponded closely with changes in membrane structural order [5,6]. However, the enzyme in carp is also subject to dietary influences [7,8] and in other vertebrates is affected by hormonal treatment. The control of desaturase expression is thus likely to be complex [9].

This enzyme is the focus of our attempts to understand the nature of the control mechanisms involved in homeoviscous adaptation. Part of our approach has been to develop an in vitro cell system with which to follow events at the cellular level during cold-adaptation. We have therefore modified published procedures for the isolation of carp hepatocytes and have studied the expression of the enzyme over 4 days of culture using enzyme activity measurement, immunoassay of desaturase protein and Northern analysis of desaturase mRNA. Surprisingly, we were unable to demonstrate a cold-induction of activity though we have detected a more profound change in desaturase expression with culture at warm temperatures.

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2. Materials and methods

2.1. Materials

Carp (*Cyprinus carpio* L., 0.4–0.5 kg) were obtained commercially and acclimated in the laboratory at 30 ± 0.2°C for at least 4 weeks. Fish were fed ad libitum once daily with trout pellets (15% crude oil (36% monounsaturated and 35% polyunsaturated), 45% crude protein, Trouw (UK), Preston, UK). All inorganic salts (analytical grade) were purchased from Merck (Lutterworth, UK). Collagenase (type A) was purchased from Boehringer-Mannheim (Lewes, UK). Foetal calf serum was purchased from Flow Laboratories (Irvine, UK). Antibiotic-antimycotic solution was purchased from Sigma Chemical Co. (Poole, UK). Restriction enzymes were purchased from Promega Corp. (Southampton, UK). All other chemicals, media and media supplements were purchased from Sigma, and were of cell culture or molecular biology grade. Primary anti-rat desaturase antibody and the plasmid expression vector, pDs3-358, were a gift from P. Strittmatter (University of Connecticut Health Centre, USA).

2.2. Preparation of carp hepatocytes

Hepatocytes were isolated by a modification of the two-step hepatic perfusion procedure described by Seglen [10,11]. All manipulations were performed in a sterile field provided by a laminar air flow cabinet, and all solutions were sterilised by membrane (0.2 μm) filtration. A constant temperature of 30°C was maintained during the isolation procedure by pre-heating all solutions in a water bath and warming the specimen using a heating mat under and a foil blanket above. The temperature of the specimen was monitored using a digital thermometer equipped with a thermistor probe. Animals were pithed, swabbed liberally with 70% ethanol, and the coeliaco-mesenteric artery cannulated [12]. Perfusion was performed at a constant rate of 12 ml min^{-1}. Open circuit perfusion was initiated with 100 ml of primary perfusion saline (in mM: NaCl, 108; KCl, 6; Na_2HPO_4, 0.5; KH_2PO_4, 0.5; $NaHCO_3$, 5; D-glucose, 5; *N*-(2-hydroxyethyl)piperazine-*N'*-(2-ethanesulfonic acid) (HEPES), 20; EDTA, 2; pH 7.6) causing the liver to swell. At this point the Cuvier sinus was punctured to allow blood and perfusate to escape. 50 ml saline (in mM: NaCl, 108; KCl, 6; $MgSO_4$, 1; Na_2HPO_4, 0.5; KH_2PO_4, 0.5; $NaHCO_3$, 5; $CaCl_2$, 4; D-glucose, 5; HEPES, 20; pH 7.6) containing 0.04% (w/v) collagenase, was then perfused at the same rate until the cannulum was empty. When perfusion was complete, the liver was removed from the animal, chopped into small pieces, and the cells separated by repeated aspiration through a Pasteur pipette tip. The resulting suspension was filtered through a 100 μm mesh to remove connective tissue and large clumps of cells and centrifuged at 100 × *g* for 5 min at 30°C. Cells were washed twice with the saline and once with Liebovitz L-15 medium (20 mM HEPES (pH 7.6), diluted with deionised water to 270 mosM kg^{-1}), and finally resuspended in L-15 supplemented with antibiotic/antimycotic solution (final concentration: 100 IU ml^{-1} penicillin, 0.1 mg ml^{-1} streptomycin, 0.25 μg ml^{-1} amphotericin B). Cells were counted in a haemocytometer, brought to a density of $0.75–1.0 \cdot 10^6$ viable cells per ml in L-15 media, with or without supplements, and plated on 60 mm- or 100 mm-diameter Primaria culture dishes (Beckton Dickinson, Cowley, UK) with a volume of 5 ml cell suspension per 25 cm^2 culture dish surface area. After plating, the cells were maintained in an incubator at 30°C under humidified air. Culture media were changed after 24 h in culture and subsequently every 48 h. When necessary cells were cooled in a programmable, refrigerated incubator. Cooling regimes for experiments are given in the figure legends.

2.3. Cell viability assays

The viability of isolated cells was assessed by trypan blue dye exclusion and succinate stimulation of cellular respiration ('SSI' [13]). Respiratory rates were measured in a microrespirometer cell equipped with an E5046 oxygen electrode (Radiometer, Copenhagen, Denmark) connected to an oxygen meter (Model 781, Strathkelvin Instruments, Glasgow, UK).

2.4. Rat desaturase induction

Rat Δ^9-desaturase was induced by the fasting-refeed method of Oshino [14,15].

2.5. Serum preparation

In some experiments the cultured hepatocytes were supplemented with serum, either foetal calf serum (FCS) or carp. Commercial FCS was heat-inactivated at 56°C for 30 min, sterile filtered through a 0.22-μm filter and aseptically dispensed into sterile containers. Carp serum was obtained from whole blood collected from either long-term warm-acclimated fish ('warm' serum) or cold-acclimated fish on day 4 of the acclimation procedure ('cold' serum). Blood was allowed to clot overnight at 4°C before separation of the serum, which was treated as described for FCS. All sera were stored at −80°C.

2.6. Fatty acid supplementation

Fatty acids were added to cell cultures at concentrations up to 100 μM as bovine serum albumin (fraction V) complexes. Fatty acid impurities were removed from BSA by charcoal treatment [16]. Oleic acid/BSA complexes were prepared according to the method of Mooney and Lane [17], and PUFA/BSA complexes according to the

method of Spector et al. [18]. Cultures were fed by total replacement of media after 24 h and every 48 h thereafter.

2.7. Electron microscopy

Isolated hepatocytes were fixed overnight in 3% glutaraldehyde at 4°C, washed in 100 mM sodium cacodylate, pelleted and stained in 1% osmium tetroxide for 2 h. After further washing and dehydration the pellet was embedded in Spurrs resin and sections (approx. 90 nm) were cut using an Ultra-Cut E microtome (Reichert, Germany) and displayed on a Zeiss EM10CR transmission electron microscope.

2.8. Isolation of hepatocyte microsomes

Microsomes were prepared from isolated hepatocytes by a modification of the method of Legrand and Bensadoun [19]. Cells were dislodged from the culture dishes by repeated pipetting. A portion of the resulting cell suspension was reserved for cell counts and viability assessment whilst the remainder was transferred to a centrifuge tube and the cells centrifuged for 5 min at $100 \times g$. The pelleted cells were re-suspended in 10 ml of 0.5 M NaCl/10 mM HEPES (pH 7.6) and washed once more. The packed cell pellet was resuspended in 500–800 μl of a 250 mM sucrose/10 mM HEPES buffered solution (pH 7.6) and sonicated (Soniprep 150, MSE, Crawley, UK) at 10 μm peak-to-peak amplitude for 15 s. The sonicated solution was then centrifuged for 15 min at $10000 \times g$ and 2°C and the supernatant taken as the microsome preparation.

2.9. The Δ^9-desaturase assay

Activity of Δ^9-desaturase was measured by a modification of the method of Leifkowith [20]. Final concentrations in the reaction tube were: potassium phosphate buffer (pH 7.6) 80 mM; NADH 1 mM; palmitoyl-CoA 12.5 μM which was trace labelled with [1-^{14}C]palmitoyl-CoA (≈ 0.14 μCi per ml reaction mixture). The reaction was initiated by the addition of microsomes (1–2 mg protein per ml of reaction mixture) and the tubes incubated at 30°C.

For each time point, a 0.2-ml aliquot of the reaction mixture was removed and added to 0.2 ml of 10% ethanolic KOH with vigorous mixing to stop the reaction. The resulting mixture was saponified at 70°C for 1 h, then acidified with an equal volume of 10% H_2SO_4. Fatty acids were extracted by the addition of 2 ml of hexane followed by rigorous mixing. The resulting emulsion was gently centrifuged and the organic upper layer containing the fatty acids transferred to a fresh tube. The extraction procedure was repeated twice, and the pooled extracts dried under N_2. Fatty acid methyl esters (FAMEs) were prepared by heating in a boiling water bath for 5 min with 1 ml BF_3-methanol complex [21] in Pyrex-glass screw-capped tubes sealed with a Teflon-lined cap. FAMEs were cooled for 2–3 min at room temperature before the addition of 1 ml of water to each tube, and extraction of the methyl esters with three 2-ml aliquots of hexane. Appropriate saturated and monounsaturated FAMEs were added as internal standards and the fatty acids separated by argentation TLC on 10% $AgNO_3$ plates developed with hexane/diethyl ether (92:10 v/v).

Following their visualisation by 2,7-dichlorofluorescein (0.2% w/v in 95% ethanol), the spots corresponding to the saturated and monoene fractions were separately scraped off the plates directly into plastic vials and subjected to liquid scintillation counting (2000CA liquid scintillation counter, Packard, Pangbourne, UK; Aqua-Luma Plus scintillation fluid, Lumac, Schaesberg, Netherlands) using quench correction. Product formed for each time point was calculated by dividing the radioactivity found in the monoene esters by the sum of radioactivities found in both monoene and saturated esters (i.e., percent substrate converted) and the 4–5 time points were used to calculate the initial rate of product formation. Activities were normalised to the amount of microsomal protein present in the assay to give nmol/min per mg of microsomal protein.

Frequently insufficient tissue was available to permit triplicate analysis of desaturase activity. However, on those occasions when triplicate analysis was performed, the standard deviation of desaturase activity measurement was 7–20% of the mean.

2.10. Membrane fatty acid analysis

Phospholipids were extracted from isolated microsomes according to the method of Bligh and Dyer [22]. Phospholipid classes were fractionated by single-dimension TLC, phosphatidylcholine and phosphatidylethanolamine classes eluted, FAMEs prepared using boron trifluoride-methanol and analysed by gas-liquid chromatography, all as described previously [23].

2.11. Western immunoassay of desaturase levels

Protein electrophoresis in sodium dodecyl sulfate and 12.5% acrylamide gels and immunoblotting were performed according to the methods described by Harlow and Lane [24] using the Mini-Protean apparatus (Bio-Rad).

2.12. Northern blot analysis

Total RNA was prepared from cultured hepatocytes according to the method of Chomzynski and Saachi [25] with minor modifications for the removal of glycogen [26]. We typically obtained 10–12 μg total RNA per million cells. Poly(A)$^+$ RNA was selected by batch affinity chromatography with oligo(dT) cellulose, purified as described [26] and stored at −80°C at a concentration of 1 mg ml^{-1}.

The poly(A)$^+$ RNA (2–5 μg) was electrophoresed in a formaldehyde-denatured agarose gel before capillary transfer to Immobilon-N PVDF membrane (Millipore) in the presence of high salt (20 × SSPE). The membranes were then baked at 80°C following the manufacturer's instructions. 1 × SSPE had the following composition: 0.15 M NaCl; 0.01 M $NaH_2PO_4 \cdot 2H_2O$; 1 mM $Na_2EDTA \cdot 2H_2O$. Hybridisation was performed, for 16–18 h, to the ^{32}P-labelled (specific activity approx. 10^8 dpm/μg) *Bam*HI/*Eco*RI insert from pDs3-358 DNA [27] using moderate stringency conditions of 30°C and 50% formamide/5 × SSPE/5 × Denhardt's reagent/1% SDS/100 μg per ml sonicated calf thymus DNA, after a minimum 6 h prehybridisation in the same solution at 42°C. The double-stranded template was labelled by random priming (Stratagene Prime-It II kit) and labelled probe separated from unincorporated nucleotides by Sephadex chromatography (Stratagene NucTrap push columns). After hybridisations, the membranes were washed as specified by the manufacturer, but only up to 0.1 × SSPE/1% SDS at 30°C, and autoradiographs of the wet blots obtained after overnight exposure at −80°C with one intensifying screen. Approximations of transcript size were made from a co-electrophoresed RNA ladder (Promega).

3. Results

3.1. Hepatocyte yield and viability

Hepatocyte yield was 49.0 ± 11.8 (mean ± S.E., $n = 6$) and 47.8 ± 7.9 ($n = 5$) million viable cells/g of liver for male and female fish, respectively. Examination of freshly isolated hepatocytes in vitro revealed highly birefringent single cells with internal birefringent corpuscles [28]. Self-aggregation of hepatocytes was observed after 24 h of culture, although the extent and rate of aggregation was greatly stimulated by the presence of a serum supplement. In contrast, cells cultured with no foetal calf serum supplement appeared morphologically similar to freshly isolated cells. They adhered to the culture dish but little cell–cell attachment was evident. By the second day of culture the cells had begun to join together as strings of cells, and these progressed by the third day into small clumps, although never as large and dense as those formed in serum-supplemented cultures.

3.2. Cell ultrastructure during culture

Freshly isolated hepatocytes possessed well formed and apparently undamaged intracellular organelles and large homogeneous areas containing glycogen particles, as has been observed previously [12,28,29] (Fig. 1A). Cell sections also contained 4–10 large membrane-bound lipid droplets [29]. With culture over 4 days, some cells developed blebs from the plasma membrane which were filled with glycogen particles or, less frequently, intracellular organelles. The abundance of cells displaying blebs increased by the fourth day in culture, and these were accompanied by a marked reduction in the abundance and size of lipid droplets and the appearance of cytoplasmic spaces devoid of particles or vacuoles (Fig. 1B). No obvious differences in ultrastructure were observed between cells cultured in the presence or absence of foetal calf serum.

The viability of cultured cells, as determined by trypan blue staining, remained greater than 95% during the entire 4-day culture period. In addition, hepatocytes consistently displayed SSIs of 1.0–1.3 during this period indicating their sustained viability. However, the number of viable cells declined over a five day period (Fig. 2). During the first 24 h of culture, 36.4 ± 3.4% (mean ± S.E., 11 experiments) of the cells were lost. After 4–5 days, 31.8 ± 6.6% (4 experiments) cells were lost which is not significantly different from the day 1 value. We conclude that cell survival remained reasonably stable up to the fourth or fifth day of incubation. Neither the absence of serum

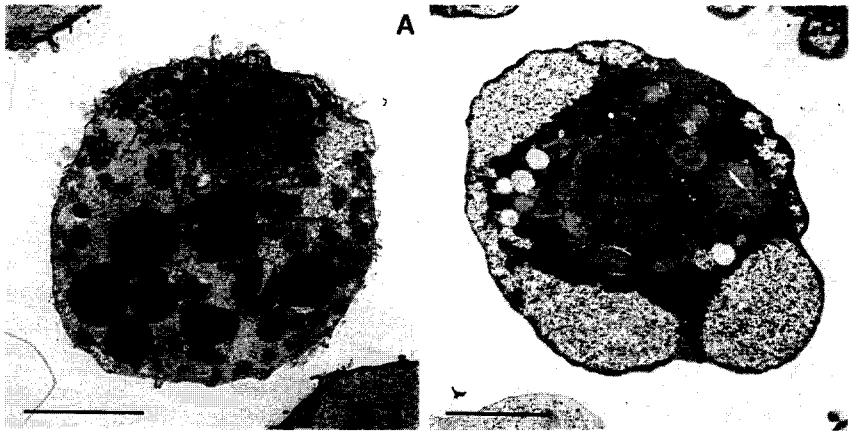

Fig. 1. Electron micrographs of carp hepatocytes after 24 h (A) and 4 days (B) in vitro culture. The bars represent 5 μm.

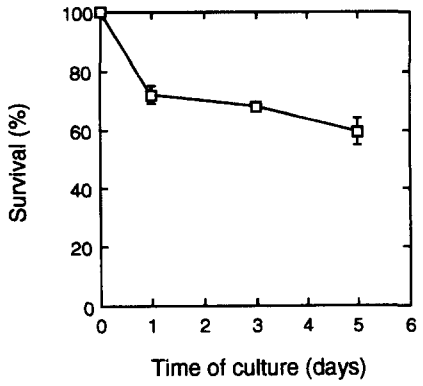

Fig. 2. Survival of a preparation of carp hepatocytes cultured at 30°C over a 5-day incubation period with 5% FCS supplement. Values represent means ± S.E. of four separate cultures originating from the cannulation of a single liver. Similar time-courses were observed on at least five separate occasions.

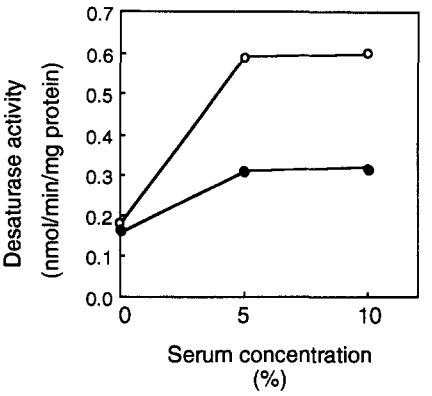

Fig. 4. Effect of foetal calf serum concentration and incubation temperature on the induction of Δ^9-desaturase activity after 24 h in culture. Cells were cultured at either 30°C (open circles) or 10°C following cooling from 30°C at 2°C/h initiated at the start of culture (closed circles).

supplements nor the temperature of incubation affected the number of viable cells remaining in culture (data not shown).

3.3. Desaturase activity

Fig. 3 shows that incubation of hepatocytes at 30°C (5% foetal calf serum supplementation) for up to 4 days increased microsomal desaturase activity by ≈ 9-fold. The increase was a highly reproducible phenomenon; in three similar experiments the mean fold increase in activity was 7.1 ± 2.1 (mean ± S.E.) after day 2 and 10.3 ± 1.6 after day 4. Fig. 4 shows that the increase in desaturase activity was greater in the presence of foetal calf serum supplement than in its absence, with 5% being sufficient for maximal activity after 2 days in culture. However, in contrast to our expectations, transfer of cells to 10°C caused a smaller increase in desaturase activity compared to cells maintained at 30°C. Thus, acclimation to a lower incubation temperature in itself did not induce higher levels of desaturase activity. In a separate experiment (data not shown), we have demonstrated that carp serum isolated from carp acutely transferred to and maintained at 10°C for 4 days did not increase the desaturase activity of 30°C-cultured hepatocytes any more than that of control cultures incubated in the presence of serum from long-term 30°C-acclimated carp.

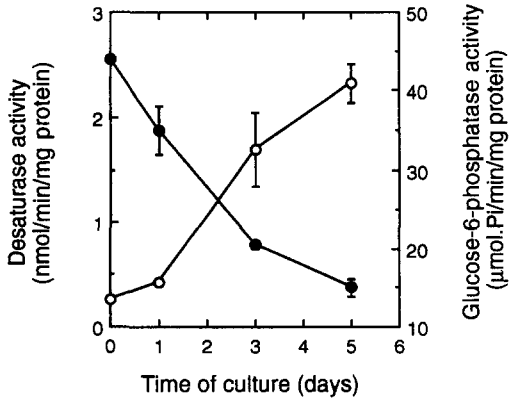

Fig. 3. Induction of Δ^9-desaturase activity (open circles) in hepatocytes isolated from 30°C acclimated carp and cultured at the same temperature over 5 days with 5% FCS supplement. Similar time-courses were observed on at least five separate occasions (see text). The graph also shows changes in the specific activity of the glucose-6-phosphatase (closed circles). Values are the mean ± S.E. of four cultures originating from a single liver.

An important question is whether the increase in desaturase activity was specific to that enzyme or whether it reflects a general increase in the activities of enzymes of intermediary or lipid metabolism. To address this we have measured the activity of a marker enzyme for gluconeogenesis, glucose-6-phosphatase (G6Pase), during the long-term primary culture of carp hepatocytes. The activity of this enzyme has been shown to vary only slightly following the acute cold transfer of whole carp [8]. Fig. 3 shows that whilst the desaturase activity increased as a function of culture time, the activity of the G6Pase was reduced by over 60% after 5 days of culture. This suggests that increased enzymatic activity during culture is not a general phenomenon.

3.4. Fatty acid composition

Table 1 shows the changes in the fatty acid composition of the major membrane phosphoglycerides, phosphatidylcholine (PC) and phosphatidylethanolamine (PE), of hepatocyte microsomes during the 4-day culture period when desaturase activity was increasing. The complex fatty acid profiles have been described by two indices, namely the ratio of saturated to unsaturated fatty acids (the saturation ratio) and the number of double bonds in 100 weight percent of fatty acids (the unsaturation index, UI). These indices emphasise different aspects of the overall fatty acid composition [23].

In phosphatidylcholine there was a progressive and substantial decrease in the proportion of 16:0 and 22:6($n-3$) with time of culture. The 40% decrease in 22:6($n-3$) was dramatic and was equivalent to nearly 16% of the total fatty acid composition after 4 days in culture. This reduction corresponded with an equally large increase in 18:1($n-9$), and a much smaller increase in 18:1($n-7$), with the result that the saturation ratio remained stable over the culture period. However, the decrease in 22:6($n-3$) accounted for a large reduction in UI. The largest changes in the proportions of 18:1($n-9$) and 22:6($n-3$) came between the second and fourth day in culture.

Similar but less dramatic changes were observed in PE, with large decreases in 16:0 and 22:6($n-3$). An increase in 18:1($n-9$) also occurred, with the greatest change after only 2 days in culture. By contrast 16:1 increased in PE whereas, if anything, there was a reduction of this species in the phospholipids of PC. Again, the saturation ratio remained relatively constant. In contrast to PC, the unsaturation index for PE remained almost constant. This was the result of the less dramatic reduction in 22:6($n-3$), and to a lesser degree, the incorporation of an increased amount of palmitoleic acid into PE. In both PE and PC there was a noticeable increase in 20:2($n-6$)/20:3($n-9$), especially between day 2 and 4.

3.5. Immunological analysis of desaturase protein levels

Immunoblots of carp and rat microsomal protein using the anti-rat Δ^9-desaturase antibody are presented in Fig. 5a. An immunopositive band of 33.8 kDa was detected in liver microsomal proteins from acutely in vivo cold-treated carp but was absent from the microsomes of control carp held throughout at 30°C. A strongly immunopositive band of approximately 39.7 kDa was detected in liver microsomes of diet-induced rats and a much weaker band was evident in microsomes from control, non-induced rats.

Fig. 5b shows the immunoblots of microsomal protein prepared from cultured hepatocytes. Microsomes from freshly isolated hepatocytes showed no antigen band; however, with progressively increasing culture time, a band of increasing intensity was observed at constant protein loading. This band was of an identical molecular mass to that of the in vivo-induced carp immunopositive protein. Fig. 5c shows a densitometric scan of the immunopositive band indicated in Fig. 5b. Band intensity increased up to ≈ 10-fold, particularly between days 3 and 4 of culture, compared with the 9-fold increase in Δ^9-desaturase activity over the same time period (Fig. 3).

3.6. Northern analysis

The results of probing RNA extracted from cultured hepatocytes with the radiolabelled rat Δ^9-desaturase cDNA probe are shown in Fig. 6. Hybrids were not observed in RNA extracts from freshly prepared hepatocytes. However, RNA from cells cultured for 2 days revealed a single hybridisation product approximately 2800 nucleotides (nt) long. Increased amounts of the same hybrid was observed from cells cultured for 4 days.

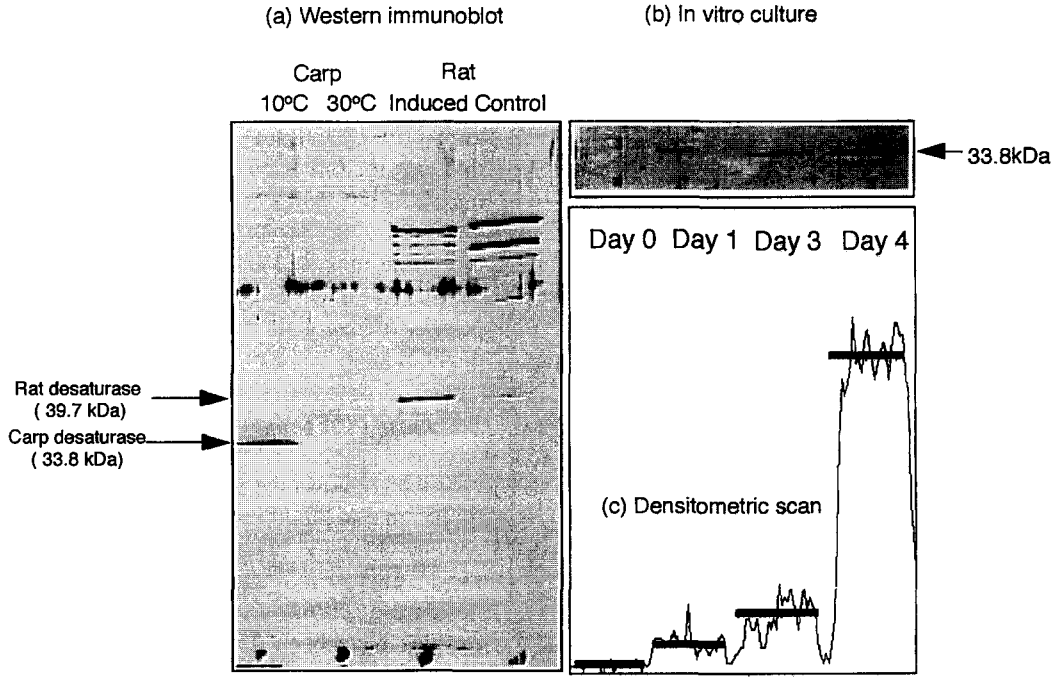

Fig. 5. Western immunoblot of desaturase levels in carp. (a) In vivo acute temperature-induced and 30°C maintained control carp and diet-induced and control rat hepatic microsomal protein. Arrows indicate the immunopositive bands corresponding to carp and rat desaturase. (b) The effects of hepatocyte culture on the expression of microsomal Δ^9-desaturase. Cells were cultured for the indicated number of days at 30°C with 5% FCS. 20 μg protein per well. The lower panel (c) shows a densitometric scan across scanned images of the immunopositive bands shown in a using NIH Image software. The level of the plot indicated by the horizontal bars represents the relative amount of immunopositive reaction in each lane.

Table 1
Fatty acid composition of microsomal PC and PE from hepatocytes cultured at 30°C for up to 4 days

Fatty acid	Day 0	Day 2	Day 4
Phosphatidylcholine			
14:0	0.5 (0.1)	0.3 (0.1)	0.7 (0.2)
15:0	0.5 (0.2)	0.2 (0.0)	0.0 (0.0)
NI	2.5 (1.9)	1.4 (0.1)	2.7 (0.3)
16:0	21.7 (1.4)	18.7 (2.0)	16.8 (2.0)
16:1	4.4 (3.3)	1.8 (0.3)	1.6 (0.4)
18:0	4.2 (0.7)	3.8 (0.6)	4.1 (0.3)
18:1($n-9$)	11.6 (0.4)	16.7 (5.7)	27.6 (0.7)
18:1($n-7$)	2.6 (0.3)	3.3 (0.4)	4.7 (1.3)
18:2($n-6$)	1.5 (0.2)	1.3 (0.6)	1.2 (0.3)
18:3($n-3$)	0.6 (0.1)	0.7 (0.1)	0.8 (0.2)
20:1($n-9$)	1.6 (0.1)	1.2 (0.3)	1.8 (0.1)
20:2($n-6$)/20:3($n-9$)	0.0 (0.0)	0.6 (0.2)	5.7 (0.6)
20:4($n-6$)	2.0 (0.3)	1.2 (0.3)	1.6 (0.2)
20:5($n-3$)	3.5 (0.6)	6.7 (6.1)	0.7 (0.1)
22:5($n-3$)	1.9 (0.3)	1.3 (0.2)	0.5 (0.1)
22:6($n-3$)	38.7 (4.9)	34.3 (6.8)	23.1 (0.2)
Sat/unsat	0.4 (0.1)	0.3 (0.1)	0.3 (0.0)
UI	291.8 (31.3)	279.8 (21.9)	202.6 (0.4)
Phosphatidylethanolamine			
14:0	0.3 (0.3)	0.5 (0.2)	0.6 (0.1)
15:0	0.1 (0.1)	0.2 (0.1)	0.1 (0.2)
NI	2.8 (2.6)	3.6 (0.9)	5.8 (2.7)
16:0	12.0 (3.9)	7.8 (1.5)	6.1 (1.2)
16:1	0.8 (0.1)	1.1 (0.7)	5.5 (2.3)
18:0	15.7 (1.6)	12.2 (2.4)	11.9 (1.6)
18:1($n-9$)	6.1 (0.8)	11.6 (1.2)	12.8 (1.0)
18:1($n-7$)	2.6 (0.3)	4.0 (0.1)	3.7 (0.2)
18:2($n-6$)	1.7 (0.5)	1.5 (0.1)	1.4 (0.3)
18:3($n-3$)	1.0 (0.2)	1.1 (0.4)	1.4 (0.8)
20:1($n-9$)	2.5 (0.1)	2.3 (0.3)	2.7 (0.4)
20:2($n-6$)/20:3($n-9$)	0.0 (0.0)	1.4 (0.4)	3.0 (0.5)
20:4($n-6$)	2.0 (0.1)	1.9 (0.1)	4.7 (0.5)
20:5($n-3$)	2.9 (0.3)	2.5 (0.2)	1.2 (1.3)
22:5($n-3$)	1.4 (0.2)	1.4 (0.1)	1.1 (0.4)
22:6($n-3$)	41.3 (10.1)	45.8 (1.3)	35.9 (3.5)
Sat/unsat	0.5 (0.1)	0.3 (0.0)	0.3 (0.0)
UI	295.3 (60.1)	330.3 (7.2)	283.4 (21.7)

Figures represent the mean of three replicates. Standard deviation for each replicate set shown in parentheses. NI, Unidentified species. UI, the unsaturation index, is the sum of each product of weight percent of unsaturated fatty acid and the number of olefinic bonds in that species. Sat/unsat is the saturation index, and is the sum of weight percent saturated fatty acids divided by the sum of weight percent unsaturated fatty acids.

The integrity of the probe has been confirmed in our laboratory by hybridisation with mRNA of approximately 4900 nt from diet-induced rats (Tiku, P., unpublished data), which is consistent with that observed by Thiede and Strittmatter [30]. Under the medium stringency conditions used hybrids will only form if there is reasonable homology between the probe and the target sequence [31]. This, together with the observation that hybrid levels increased concurrently with desaturase activity induction, suggests that the probe was hybridising with mRNA encoding the carp Δ^9-desaturase protein.

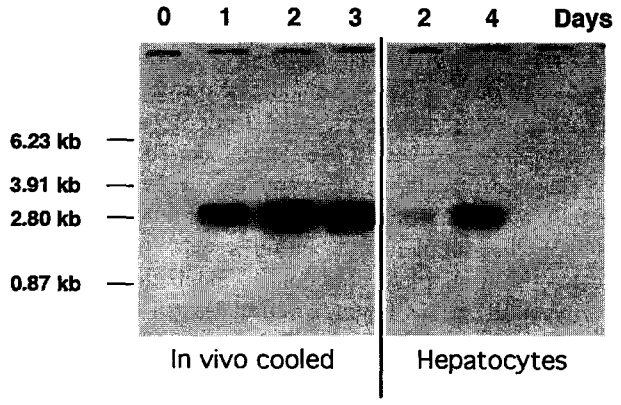

Fig. 6. Northern blot analysis of desaturase mRNA levels. Blots were probed under conditions of moderate stringency with a *Bam*HI/*Eco*RI insert isolated from rat desaturase clone pDs3-358, and radiolabelled by random-priming. In the left panel, RNA samples were isolated from carp maintained for several months at 30°C (lane 0) and at the indicated periods following initiation of cooling to 10°C (lanes 1–3). The panel on the right shows RNA samples from isothermally cultured carp liver cells. RNA sizes are indicated in kilobases.

3.7. Effects of lipid supplementation

The induction of desaturase activity during long-term primary culture might be due to a lipid deficiency in the culture medium. We have therefore supplemented cultures with fatty acids complexed with BSA. Fig. 7 shows that there was no depression of desaturase activity after long-term primary culture with increasing oleic acid concentrations up to 100 μM, desaturase activity increasing by approximately 9-fold at all concentrations supplemented. Evidently this fatty acid, a product of the desaturase reaction, has no effect on the in vitro induction of desaturase activity.

In a second experiment PUFA supplements were delivered at a final concentration in the culture media of 100 μM. Fatty acid profiles indicated a moderate degree of enrichment of the supplemented fatty acid. Thus, in linoleic

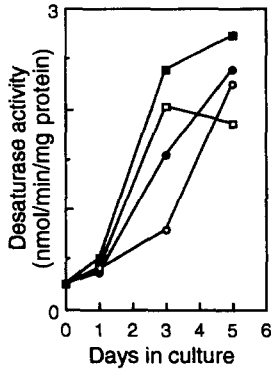

Fig. 7. Effect of oleic acid supplementation upon desaturase induction during 4-day culture. Oleic acid was provided as a complex with BSA at 0 (open circles), 25 (closed circles), 50 (open squares) and 100 μM l^{-1} (closed squares). Cells were cultured at 30°C with 5% FCS and fed by total media replacement after 24 h of culture and every 48 h thereafter.

acid-supplemented cultures the proportion of linoleic acid rose from 2.4% in freshly isolated cells to 6.9% after 4 days of culture. Similarly, linolenic acid rose from 0.3% to 2.2%, and eicosapentaenoic acid from 2.9% to 5.7% in cells supplemented with those fatty acids. 20:5($n-3$) was also elevated in cells supplemented with the precursor of this fatty acid, 18:3($n-3$). However, the desaturase activities in PUFA-supplemented cultures was similar to fatty acid-free BSA-supplemented control incubations, the mean activity in all groups having risen at least 8-fold over that of freshly isolated cells after 4 days of culture (data not shown).

4. Discussion

Teleost hepatocytes have been used widely to assess the role of direct cellular responses in whole animal temperature adaptations [32]. This is based on the assumption that the cultured cells do not suffer from pathological effects of in vitro culture or from a progressive dedifferentiation. However, we show here that although carp hepatocytes may be cultured for up to 5 days, there were noticeable changes in cell ultrastructural morphology and in the rates of lipid biosynthesis. Firstly, some cells develop membrane-bound blebs extending from the surface of the otherwise spherical cell. Secondly, there was a noticeable reduction in the number and size of the large intracellular lipid vesicles. Thirdly, there was a progressive induction of Δ^9-desaturase activity which corresponded in time course with the loss of lipid vesicles.

There are several potential mechanisms which might lead to increased desaturase activity, including changes in enzymatic activity due to post-translational modification, changes in the level of expressed desaturases due to enhanced synthesis or reduced degradation, or perhaps due to the production of a different desaturase isoform [9]. We show that the increase in desaturase activity broadly parallels the increase in immunodetectable protein which is consistent with an increase in the number of active enzymatic units rather than their molar specific activity. Furthermore, we have identified a mRNA transcript which hybridises under conditions of moderate stringency with a probe derived from the rat hepatic Δ^9-desaturase. This probe has more recently been used to screen a carp cDNA library and to isolate a clone with extensive regions of amino acid sequence homology with the published rat sequence [33]. This clone also binds to a transcript of 2.7 kb which supports the identity of the mRNA detected by the rat probe as coding for the Δ^9-desaturase. We show that this transcript was undetectable in freshly isolated hepatocytes but was detectable after 2 days of culture and continued increasing up to day 4. Thus, desaturase induction was linked to increased levels of transcript and this appears to lead to increased levels of desaturase protein. Whether this is due to increased rates of mRNA synthesis or decreased degradation has not been established, though we have recently shown that in vivo cooling of carp causes an up-regulation of transcriptional rates in isolated nuclei [33].

Exactly why desaturase transcript and protein levels increased during long-term hepatocyte culture is not clear. An obvious possibility is that the dietary provision of lipids is disturbed as a result of isolation of the cells and their culture. The carp used in these experiments were fed ad libitum with a high fat trout diet and maintained at a high water temperature for several months. As a result their carcasses were invested with extensive deposits of adipose tissue throughout the abdominal cavity and the hepatocytes possessed large numbers of large lipid vesicles. Thus at the time of isolation it seems that hepatic lipid requirements may have been provided entirely from dietary sources, and this might account for a down-regulation of desaturase expression.

By contrast, the only dietary source of lipid for cells in culture is from the foetal calf serum supplement, and lipid provision is substantially lower than in vivo. We show that hepatocytes lose their endogenous lipid stores during culture which indicates a non-steady state condition as far as lipids are concerned. Membrane-bound, triglyceride-rich vesicles (TGRVs) have also been reported in chicken hepatocytes [17] and other species, where they appear to function as storage sites for triglyceride when the rate of synthesis of triglycerides exceeds that of the other components required for the production of very low density lipoproteins (VLDLs). The loss of TGRVs during culture of carp hepatocytes may result from the continuing production of VLDLs without the corresponding synthesis of triglyceride or its provision within the foetal calf serum supplement. The increased desaturase expression, and the increased levels of de novo lipid products evident in membrane phosphoglycerides, may therefore be part of a compensatory response to these declining lipid stores and to a reduction of the intracellular fatty acid-CoA pool. In this case, we expect that other lipid biosynthetic enzymes, such as fatty acid synthase, would also be up-regulated in a coordinated response.

The phosphoglyceride fatty acid composition shows the incorporation of increased proportions of oleic acid over the culture period, this being the product of the desaturase reaction, as well as a peak that might be an elongation product of oleic acid (20:3($n-9$)). Thus, turnover of microsomal phospholipids continues during culture and the desaturase induction has a clear impact upon the composition of both the fatty acid pool and the membrane phosphoglycerides. This increase in monoenoic fatty acids was largely at the expense of 22:6($n-3$). As a result the ratio of saturated to unsaturated fatty acids, which is regarded as a useful indicator of membrane physical condition, remains relatively constant over the full time-course of the 5-day culture. However, the unsaturation index showed a signifi-

cant reduction due to the replacement of long chain polyene by the monoene.

One means of preventing a general up-regulation of de novo lipid biosynthetic capacity would be to provide sufficient quantities of an exogenous lipid source. However, we show that despite the enrichment of supplemented fatty acid into phosphoglyceride fatty acids and hence into the fatty acyl-CoA pool, this procedure did not prevent the loss of TGRVs or the induction of desaturase activity. In a number of other cell types the expression of the Δ^9-desaturase is sensitive to exogenous fatty acid supplementation. For example, in yeast the *OLE1* gene, which codes for a Δ^9-desaturase, may be transcriptionally repressed by addition to the growth medium of unsaturated fatty acids containing a double bond at the C9–C10 position [34]. Similarly, in mammalian lymphoma cells, desaturase transcription is down-regulated by addition of arachidonic acid [35]. Both of these studies have used fatty acid concentrations in the range 10–50 μM which is similar to the range of concentrations used in the present study. Interestingly, in mammalian lymphoma cells, a reduction in serum supplement produced a substantial increase in endogenous 18:1 synthesis, indicating that usual levels of serum supplement provided sufficient exogenous lipid not to require endogenous lipid biosynthesis [35].

Because of their central role in whole animal lipid turnover, hepatocytes have a much greater lipid requirement than other cell types, irrespective of whether the precursor fatty acids are provided by endogenous biosynthesis or from exogenous sources [36]. It follows that the down-regulation of de novo synthesis in hepatocytes requires substantially higher levels of dietary supply than other cell types. Indeed, in cultured chicken hepatocytes the formation of TGRVs required oleate supplement at millimolar concentrations [17], which is 10–20-fold greater than used with other cell types. Thus, our inability to suppress desaturase induction by supplementation was not necessarily due to an insensitivity to lipid supplementation but to a much higher threshold for suppression of biosynthetic capacity.

A second possible explanation for desaturase induction in vitro is that the isolation and culture of hepatocytes removes them from some systemic factor(s) which suppresses the expression of lipid biosynthetic capacity in liver. Indeed, a major question in the temperature adaptation of animals is whether the underlying cellular responses occur at the level of individual cells or requires some systemic input to initiate the response [32]. Interestingly, hepatic desaturase expression is sensitive to hormonal manipulation. For example, in chick hepatocytes, insulin and carbohydrate metabolites caused increased desaturase activity [37]. Insulin also initiates the transcriptional activation of acyl-CoA synthetase and the Δ^9-desaturase in cultured 3T3-L1 cells [38]. In the present studies, supplementation of carp hepatocyte cultures with foetal calf serum increased the magnitude of the desaturase induction, and the cells displayed hepatogenesis and a quite different morphology. We have shown that addition of serum isolated from either cold- or warm-acclimated carp did not affect the desaturase induction, making it unlikely that a serum factor was involved either in down-regulation or in inducing activity in the cold.

Finally, in vivo cooling of carp causes an activity induction of up to 15- to 30-fold [4,6]. Given the isothermal induction described above, we have investigated whether cooling of hepatocytes causes an enhanced desaturase induction compared to cells maintained at 30°C. We show, in contrast to our expectations, that cooled hepatocytes gave a smaller increase in desaturase activity than control cells held at 30°C. However, given the observed isothermal desaturase induction due to rapid losses of endogenous lipid reserves, the situation on cooling becomes rather complex. The reduced induction in the cold might be due a simple rate effect of cooling which given sufficient time would lead to an equal or greater desaturase induction than at warmer temperatures. Alternatively, cooling might lead to a slower reduction of endogenous lipid stores and hence to a slower increase in desaturase expression. There is no published information on whether in vivo cooling over several days leads to a reduction of lipid vesicles in liver parenchyma cells. Either way there is at present no evidence of a desaturase induction in response to cold which matches the in vivo response and this limits the use of a hepatocyte model for investigating the cellular and molecular mechanisms of homeoviscous adaptation.

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