Low temperature directly activates the initial glycerol antifreeze response in isolated rainbow smelt (Osmerus mordax) liver cells

Kathy A. Clow,1 K. Vanya Ewart,2 and William R. Driedzic1

1Ocean Sciences Centre, Memorial University of Newfoundland, St. John's, Newfoundland; and 2National Research Council Institute for Marine Biosciences, Halifax, Nova Scotia, Canada

Submitted 21 April 2008; accepted in final form 10 July 2008

Clow KA, Ewart KV, Driedzic WR. Low temperature directly activates the initial glycerol antifreeze response in isolated rainbow smelt (Osmerus mordax) liver cells. Am J Physiol Regul Integr Comp Physiol 295: R961–R970, 2008. First published July 16, 2008; doi:10.1152/ajpregu.90372.2008.—Rainbow smelt (Osmerus mordax) accumulate high levels of glycerol in winter that serve as an antifreeze. Liver glycogen is a source of glycerol during the early stages of glycerol accumulation, whereas dietary glucose and amino acids are essential to maintain rates of glycerol synthesis. We presently report rates of glycerol and glucose production by isolated hepatocytes. Cells from fish held at 0.4 to −1.5°C and incubated at 0.4°C were metabolically quiescent with negligible rates of glycerol or glucose production. Hepatocytes isolated from fish maintained at 8°C and incubated at 8°C produced glucose but not glycerol. Glycerol production was activated in cells isolated from 8°C fish and incubated at 0.4°C without substrate or when glucose, aspartate, or pyruvate was available in the medium. Incubation at 0.4°C without substrate resulted in similar molar rates of glucose and glycerol production in concert with glycogen mobilization. Glycogenolysis and glycerol production were associated with increases in total in vitro activities of glycogen phosphorylase and glyceraldehyde dehydrogenase. Maximal in vitro activities of hexokinase and glucokinase were not influenced by temperature, but high activities of a low-\(K_m\) hexokinase may serve to redirect glycogen-derived glucose to glycolysis as opposed to releasing it from the cells. Rates of glycerol production were not enhanced in cells from fish held at 8°C and incubated at 0.4°C with adrenergic or glucocorticoid stimulation. As such, low temperature alone is sufficient to activate the glycerol production mechanism and results in a shift from glucose to a mix of glucose and glycerol production.

glycogen phosphorylase; glyceraldehyde-3-phosphate dehydrogenase; hexokinase; glucokinase

RAINBOW SMELT (Osmerus mordax) accumulate high levels of glycerol in winter that serve as an antifreeze in plasma and all other tissues. Glycerol levels are substantially higher than in all other tissues, and a temperature decrease from 8 to −1.2°C over a period of 12 days essentially depletes glycogen stores in liver of fed fish. The rate of depletion is even faster in food-deprived animals (5). In this study, we assessed the impact of temperature on the ability of isolated liver cells to mobilize glycogen with the associated production of glycerol. The control of glycogen utilization and how it relates to glucose release in fish liver cells has been well studied. The first step in glycogen breakdown is catalyzed by glycogen phosphorylase (GPase), which is to a large extent under catecholamine control. Adrenergic stimulation results in increased percent GPase activity in association with elevated glucose release (16). Potentially relevant to understanding the processes in liver of rainbow smelt, as relates to freeze tolerance, are studies with amphibians. For instance, on the initiation of freezing, the wood frog (Rana sylvatica) produces glucose from large glycogen reserves in liver, a process that is under adrenergic control (27, 28). The situation is probably similar in the gray tree frog (Hyla versicolor), a species that produces glycogen as an antifreeze agent, since cold exposure results in increases in percent GPase activity (26). In the present study we examined the impact of temperature and adrenergic stimulation on GPase activity in isolated hepatocytes of rainbow smelt. Although there is compelling evidence that liver of rainbow smelt produces glycerol, the possibility that glucose produced by liver is also cycled to other tissues to be converted to glycerol extrahepatically cannot be ruled out. In the experiments executed, we noted that rates of glycogen depletion exceeded rates of glycerol production. We therefore assessed the rates of glucose and glycerol production by isolated liver cells again as influenced by temperature and adrenergic stimulation.

Further aspects of this study relate to control of glycerol production at the level of glyceraldehyde-3-phosphate dehydrogenase (GPDH) and the determination of whether other potential hormonal signals can enhance glycerol production. Glycerol is formed from glycogen, glucose, and amino acids via the conversion of dihydroxyacetone phosphate (DHAP) to glyceraldehyde-3-phosphate (catalyzed by GPDH) and subsequently to glycerol (4). Cytosolic GPDH activity and gene expression are increased during the active phase of glycerol accumulation in rainbow smelt following the natural thermal cycle (12, 13). GPDH activity increases in liver of fish subjected to acute decreases in water temperature (6), and GPDH mRNA decreases in fish subjected to acute temperature increase (8). GPDH activity is stimulated in rat hepatoma cell lines and trans-
formed rat hepatocytes after 3 days of in vitro culture with glucocorticoids (14), and GPDH activity and mRNA level are stimulated by glucocorticoids in the rat brain (2). Also, in mouse liver, the GPDH gene has a functional peroxisome proliferator-activated receptor (PPAR) response element in its promoter that is a direct target of PPARα (21). In this study we determined the activity of GPDH in isolated cells subjected to acute temperature decrease and the impact of glucocorticoid and PPARα stimulation on glycerol production.

In addition to glycogen, glucose is an important substrate for glycerol production by liver of rainbow smelt, as evidenced by injected radioisotopes and stable isotopes of glucose appearing in labeled glycerol (22, 32) and the necessity for continuous food intake for survival and glycerol production at low temperature (5). In this study, we assessed the enzymatic capacity for glucose phosphorylation by measuring low-Km hexokinase (HK) and high-Km glucokinase (GK) activities. GK activity, GK gene expression, and HK activity are induced by dietary carbohydrate in liver of rainbow trout (Onchorhynchus mykiss) (25), presumably in association with hormonal signaling. In this study, we assessed whether a low temperature-induced change in glucose trafficking might directly result in changes in enzyme activity patterns.

The most important findings are that low-temperature challenge alone is sufficient to activate the glycerol production mechanism (i.e., hormonal signals to liver are not a requirement) in isolated cells. Low-temperature activation of glyco-
genolysis is associated with an increase in total GPase activity, and there is a switch from glucose release at high temperature to a mix of glucose plus glycerol release at low temperature.

MATERIALS AND METHODS

Animals

Rainbow smelt (O. mordax Mitchell) were collected by seine netting from Long Harbour, Newfoundland, in October between the years 2002 and 2007, transported to the Ocean Sciences Centre, Memorial University of Newfoundland, and held in 3,000- or 1,800-
liter tanks with flow-through seawater. Fish were kept on a natural photoperiod with fluorescent lights set by an outdoor photocell and fed a diet of herring twice a week. After 4 wk while water temperature was at 8–10°C, some of the fish were separated into two netting from Long Harbour, Newfoundland, in October between the December and April, the period over which rainbow smelt normally have high levels of glycerol (12). Fish were not held past April in any given year, and a new group of animals was collected every October. Experiments were conducted over six separate years. Both male and female fish were utilized, and fish ranged in mass from 24 to 132 g. Animal protocols were approved by the Institutional Animal Care Committee, Memorial University of Newfoundland.

Hepatocyte Isolation

Rainbow smelt were randomly removed from both the warm and cold water tanks and then killed by a quick blow to the head, and the liver was immediately exposed. Hepatocytes were isolated by perfusing the liver with collagenase as described by Moon et al. (17) with minor modifications. Briefly, a retrograde perfusion was performed by cannulating the hepatic vein and nicking the venous input for drainage. Perfusion medium containing (in mM) 176 NaCl, 5.4 KCl, 0.81 MgSO₄, 0.44 KH₂PO₄, 0.35 Na₂HPO₄, 5 NaHCO₃, 10 HEPES, and 1 EGTA, adjusted to pH 7.63, was used to clear blood. This was the basic medium for all isolation and cell suspension protocols. The perfusion was carried out at room temperature at a flow rate of 1–1.5 ml/min. After 10 min, the liver was transferred to a watch glass and perfused with the above medium containing 0.3 mg/ml collagenase IV (Sigma-Aldrich, St. Louis, MO) and no EGTA. The liver was per-
fused with this recirculated collagenase medium for 25 min or until the tissue was soft. The liver was gently teased apart, and the isolated cells were filtered, washed three times in medium containing 2% BSA (fraction V, fatty acid free; ICN Biochemicals) and 1.5 mM CaCl₂, and counted using a Neubauer hemocytometer. Cell viability was assessed at this time by Trypan blue exclusion and was usually >95%. Any preparations with viabilities <90% were rejected. Cells were resuspended at a concentration of 40 × 10⁶ cells/ml. In some cases, hepatocytes from two individuals were pooled to obtain enough cells to complete one experiment. Initial (preincubation, or 0 h) hepatocyte samples were collected by adding 150 μl of the cell suspension or 6 × 10⁷ cells (corresponding to 10–30 mg of cells) to two or three microcentrifuge tubes. These tubes were centrifuged at 1,000 g for 10 min, the supernatant was transferred to a cryovial, and both the cell pellet and supernatant were snap frozen in liquid nitrogen. Samples were stored at −80°C until glycerol, glucose, glycogen content, and/or enzyme activities were measured. The remaining cell suspension was divided into 20-ml glass scintillation vials (10–30 mg of cells per vial) containing 2 ml of medium with 2% BSA and 1.5 mM CaCl₂ and incubated at either 0.4°C (cold incubation) or 8°C (warm incubation). Preliminary experiments comparing cells plated in six-well Primaria plates (Falcon) to cells plated in 20-ml scintillation vials indicated no difference in viability and glycerol production after 24, 48, and 72 h. By 72 h, hepatocytes established cell-to-cell connections and formed monolayers. Viabilities at 72 h were usually >90%, and any viabilities below this were rejected.

Experimental Protocols

Experiment 1: Linearity of glycerol production. Initial experiments were performed to determine whether glycerol was produced at a linear rate. Hepatocytes from warm or cold smelt were incubated at 0.4°C with 5 mM glucose or without substrate in the medium for 24, 48, and 72 h. At each time point, the hepatocyte suspension was transferred to separate centrifuge tubes and spun at 1,000 g for 10 min, the supernatant was collected, and both portions were frozen in liquid nitrogen for glycerol analysis.

Experiment 2: Impact of acclimation/incubation temperature and substrate availability on glycerol production. After confirming that glycerol was produced in a linear fashion by isolated cells in Experiment 1, we next determined whether supplementing the medium with potential substrates could enhance glycerol production and assessed the impact of acclimation/incubation temperatures on rates of glycerol production. Hepatocytes from warm fish were incubated at either 0.4 or 8°C in medium without substrate or in medium containing one of the following (in mM): 5 glucose, 5 L-alanine, 5 L-aspartate, or 1 pyruvate. Hepatocytes from cold fish were incubated at 0.4°C. Glycerol production and cell viability were determined after 72 h.

Experiment 3: Glycerol production, GPase, and potential adrenergic activation of glycoconjugation. Experiment 2 showed that even in the absence of substrate, hepatocytes from warm-acclimated fish produce glycerol when incubated at cold temperature. The next series of experiments examined whether glycogen was a potential source of glycerol and determined the state of GPase (EC 2.4.1.1). In addition, we assessed whether glycerol production and GPase activation state could be enhanced by adrenergic stimulation.

Samples were collected at preincubation and after 72 h of incubation with no exogenous substrate in the medium. Glycogen level was determined in cells isolated from warm-acclimated rainbow smelt and incubated at 0.4°C or, in two cases, at 8°C. Glycogen was not
measured in fish held at cold temperature, since it is known to be minimal (5). Thereafter, experiments consisted of three acclimation/incubation groups: warm fish/8°C incubation, warm fish/0.4°C incubation, and cold fish/0.4°C incubation. Cells were incubated without additional components or with 1 or 10 μM isoproterenol (a nonspecific β-agonist; Sigma-Aldrich) in the medium. Hepatocytes were assayed for glycerol production and GPase activity (total and specific) with or without additional components or with 1 or 10 μM isoproterenol in the medium. Rates of glycerol and glycerol production were determined.

Experiment 4: glycerol and glucose production. Experiment 3 revealed that decreases in glycogen could not be accounted for by increases in glycerol alone. This led to studies that included glucose measurements. Samples were collected at preincubation and after 72 h of incubation with no exogenous substrate in the medium. Experiments consisted of three acclimation/incubation groups: warm fish/8°C incubation, warm fish/0.4°C incubation, and cold fish/0.4°C incubation. Cells were incubated without additional components or with 1 or 10 μM isoproterenol in the medium. The level of isoproterenol used was based on the review of Fabbri et al. (9), who concluded, on the basis of many studies, that the general concentration of catecholamine required to stimulate maximal glucose production by isolated fish hepatocytes is in the 10 μM range, although much lower concentrations may be effective.

Experiment 5: GP DH, HK, and GK activity. The aim of this experiment was to assess whether enhanced glycerol production was associated with increases in the activity levels of key enzymes. Cells were collected at preincubation and after 72 h of incubation with 5 mM glucose in the medium. Experiments involved fish acclimated to warm temperature with hepatocytes from the same population of fish incubated at 8 and 0.4°C. Glycerol production was assessed along with GPDH, HK (EC 1.1.1.8), HK (EC 2.7.1.1), and GK (EC 2.7.1.2) activities. Six whole livers from other fish were also collected and frozen at this time.

Experiment 6: glucocorticoid and PPAR activation. We assessed whether the presence of glucocorticoids or a putative PPAR activator could enhance glycerol production. Cells were collected at preincubation and after 72 h of incubation with 5 mM glucose in the medium. Experiments consisted of three groups: warm fish/8°C incubation, warm fish/0.4°C incubation, and cold fish/0.4°C incubation. Glycerol production was assessed without any further incubation. Cells were incubated without additional components or with 1 or 10 μM isoproterenol in the medium. The level of isoproterenol used was based on the review of Fabbri et al. (9), who concluded, on the basis of many studies, that the general concentration of catecholamine required to stimulate maximal glucose production by isolated fish hepatocytes is in the 10 μM range, although much lower concentrations may be effective.

Analysis

Glycerol, glycogen, and glucose. Both the medium and the cell pellet were analyzed for glycerol in duplicate. Glycerol was directly determined from the media samples using a free glycerol kit (F6428; Sigma-Aldrich). Cell pellets were dissolved in 9 volumes of 6% perchloric acid, the homogenate was neutralized with 20% KOH and spun at 10,000 g for 10 min, and the supernatant was assayed for glycerol. Glycerol was directly determined in the medium and pellet were summed and expressed per gram of cell. The rate of glycerol or glucose production was calculated by subtracting postincubation from preincubation levels and dividing by time.

For glycogen analysis, 9 volumes of 30% KOH were added to each cell pellet and boiled for 10 min. Glycogen was precipitated by adding 0.15 ml of 2% aqueous Na2SO4 and 1.0 ml of absolute ethanol. After the mixture was centrifuged for 10 min, the supernatant was decanted. The pellet was washed with 1 ml of 66% ethanol and dissolving in 0.5 ml of warm distilled water. An equal volume of 1.2 M HCl was then added, and the sample was heated in a boiling water bath for 2 h (31). Hydrolysates were frozen in liquid nitrogen and stored at −80°C until analyzed for glucose content.

Assay conditions for glucose released during glycogen measurements and free glucose produced during hepatocyte incubation were based on a procedure modified from Bergmeyer et al. (1). Briefly, an assay medium was prepared containing (in mM) 250 imidazole, 5 MgSO4, 10 ATP, and 0.8 NADP+. A 25- or 50-μl aliquot of the sample was added to a 96-well plate and diluted 1:10 or 1:5 with the assay medium. Twenty-five microliters of 10 U/ml glucose-6-phosphate dehydrogenase (G6PDH) were added to remove any endogenous glucose-6-phosphate (G6P). Absorbance was read at 340 nm on a DTX 880 plate reader (Beckman Coulter) after 10 min. Twenty-five microliters of 10 U/ml HK were then added, and the absorbance was read after 45 min.

Enzyme assays. For GPase, cell pellets were homogenized in 19 volumes of ice-cold extraction buffer containing (in mM) 50 imidazole, 5.0 EGTA, 2.0 EDTA, 30 mercaptoethanol, 100 Naf, and 0.1 PMSF, pH 7.4 at 4°C. Samples were incubated on ice for 30 min, and the supernatant was assayed for GPase a and total (a + b) activity.

Assay conditions were based on a procedure modified from Joanisse and Storey (11). Total GPase was measured in 50 mM potassium phosphate buffer (pH 7.0) containing (in mM) 0.4 NAD+, 0.010 glucose-1,6-bisphosphate, 0.25 EDTA, 15 MgCl2, 1 U/ml G6PDH, 1 U/ml phosphoglucomutase, and 1.6 AMP. The reaction was initiated by adding 2 ml/glucose (oyster muscle, dialyzed; Sigma-Aldrich). Control and reaction rates were determined separately. GPase a activity was measured in the absence of AMP. The percent GPase a activity was calculated by dividing GPase a by total GPase (a + b) activity.

For GPDH, HK, and GK activities, frozen cell pellets or pieces of liver were homogenized in 9 volumes of ice-cold extraction buffer containing (in mM) 50 triethanolamine, 1.0 DTT, and 5.0 EDTA, pH 7.6 at 4°C. The homogenate was spun at 1,000 g for 5 min, and an aliquot of the supernatant was diluted 1:20 for GPDH determination, and the rest was used for HK and GK determination. GPDH assay conditions included (in mM) 20 imidazole, pH 7.2 at 20°C, 0.15 NADH, and 2.0 DHAP, which was used to initiate the reaction. HK and GK assay conditions (in mM) 50 triethanolamine, pH 7.6 at 20°C, 0.2 NADP+, 8.0 MgCl2, 7.0 ATP, and 0.4 U/ml G6PDH. The reaction was initiated by 1 mM glucose for HK (low Km) determination and 100 mM glucose for total glucose phosphorylation activity. Control rates were determined after the addition of the substrate. In addition, glucose dehydrogenase (G1DH; EC 1.1.1.47), a microsomal enzyme in fish liver, was measured to correct GK activities in these frozen samples. G1DH can introduce significant bias into GK measurements in frozen tissues; thus GK activity was calculated as the total glucose phosphorylation activity minus low-Km HK activity minus one-third of G1DH activity (20). G1DH assay conditions were similar to total glucose phosphorylation activity conditions except that ATP and G6PDH were omitted. The above homogenates were also assayed for protein by using a Coomassie (Bradford) protein assay kit (23200; Pierce, Rockford IL).

Enzyme activities were determined at 340 nm on a Beckman DU640 spectrophotometer, and activity was calculated on the basis of a millimolar extinction coefficient of 6.22. All enzyme activities were determined at 20°C to facilitate analysis at higher activity levels, since we were primarily interested in relative, not absolute, activities during exposure of fish to decreased temperature. This was considered acceptable with the assumption that enzyme activity is proportional to enzyme content. Enzymes activities were expressed as micromoles per minute per each gram of cells or milligram of protein.

Data Analysis

Preincubation versus 72-h incubation values or treatment effects after 72 h of incubation were compared using Wilcoxon signed rank test.
tests when paired samples from the same cell preparation were available. The Mann-Whitney U-test was utilized in situations where some, but not all, of the values were obtained from the same population of cells. The Kruskal-Wallis test was used to determine the statistical differences when three groups were compared. This was followed by the Mann-Whitney U-test to identify between-treatment differences. Student’s t-test was used to compare GPDH, HK, and GK activity levels in whole liver with activity levels in freshly isolated hepatocytes from different fish. P ≤ 0.05 was considered to be statistically significant for all studies. A statistical software package (SPSS) was used for all statistical analysis.

RESULTS

Initial Glycerol Levels and Linearity of Glycerol Production

Total glycerol measured in preincubation cells isolated from warm smelt was 11.41 ± 1.28 μmol/g (n = 17; data from experiment 2 only). This level is similar to that found in whole livers from smelt before the antifreeze response (12) and in smelt maintained at 8°C (6). Glycerol level in preincubation hepatocytes from cold fish was 8.63 ± 0.97 μmol/g (n = 8), a value lower than expected compared with levels found in vivo at that acclimation temperature. Analysis of the postwash solution indicated that most of the glycerol in the hepatocytes was lost during washing and/or liver perfusion. Attempts to increase the osmolality of the perfusion and washing solutions (to equal the osmolality of the in vivo liver) resulted in unacceptable viability.

The production of glycerol, assessed as the sum of glycerol in the cells plus glycerol released into the incubation medium, was linear for at least 72 h (Fig. 1). In groups that showed elevated levels of glycerol, ~90% of the total glycerol was found in the incubation medium. Subsequent experiments used 72-h incubations.

Hepatocytes From Warm Fish Produce Glycerol at Low Temperature

Total glycerol concentrations at preincubation and after 72 h of incubation, either without substrate or with (in mM) 5 glucose, 5 alanine, 5 aspartate, or 1 pyruvate in the incubation media, are compared in Fig. 2. Fish held at warm temperatures and not yet engaged in enhanced glycerol production in vivo did not show an increase in glycerol levels when hepatocytes were isolated and incubated at 8°C (Fig. 2A). Incubation without substrate or with alanine in the medium resulted in a small but significant decrease in total glycerol levels. In contrast, when hepatocytes from warm fish were incubated at 0.4°C for 72 h, glycerol synthesis was activated (Fig. 2B). Significant increases in glycerol occurred when either glucose or pyruvate was added to the medium (104.8 and 76.8 μmol/g, respectively, compared with a preincubation level of 11.2 μmol/g). With incubation without substrate or with aspartate in the medium, there was a tendency for an increase in glycerol production with respect to preincubation levels (P values were 0.059 and 0.052, respectively). No significant changes in glycerol were seen when hepatocytes from cold fish were incubated at 0.4°C except when glucose was added to the medium (Fig. 2C). Under these conditions, glycerol levels increased from the preincubation values of 8.63 to 17.64 μmol/g.

Rates of glycerol production were calculated to compare glycerol production at different acclimation/incubation temperatures (Table 1). The rate of glycerol accumulation at 0.4°C incubation temperature with either glucose or aspartate in the medium was significantly higher in warm-acclimated than in cold-acclimated fish. In the absence of exogenous substrate, the average glycerol production was higher in cells from warm-acclimated than from cold-acclimated fish when tested at 0.4°C; however, in this experiment, this difference was not statistically significant. Glycerol production by cells from warm fish incubated at 0.4°C was significantly higher than by cells from warm fish incubated at 8°C with glucose, aspartate, or no substrate in the medium. There were no significant differences in rates of glycerol production between groups when the medium contained either alanine or pyruvate (data not shown).

Glycerol Production is Associated With an Increase in Total GPase

Initial glycogen levels were measured from hepatocytes prepared from warm-acclimated smelt. When these cells were incubated in the absence of substrate at 0.4°C for 72 h, there was a significant decrease in glycogen content (from 214.9 ± 79.8 to 17.4 ± 12.4 μmol glucosyl units/g; n = 7). Cells from two of these warm fish were also incubated at 8°C and showed a similar decrease in glycogen levels (to 21 and 42 μmol glucosyl units/g).

Hepatocytes from warm fish incubated at 0.4°C produced significant amounts of glycerol after 72 h compared with the preincubation level (180.3 ± 31.64 vs. 11.37 ± 1.69 μmol/g) (Fig. 3). The direction of change in glycogen level in this study was consistent with the previous experiment, although the magnitude of change was greater. Preparations of cold fish/0.4°C or warm fish/8°C cells did not produce glycerol in the absence of substrate. Instead, cells from warm fish incubated at 8°C showed significant glycerol decrease. When values were expressed as rates, glycerol production in warm fish/0.4°C cells was significantly higher than glycerol production in warm fish/8°C or cold fish/0.4°C cells (Table 2). If all of the glycogen in the cells from warm fish incubated at 0.4°C was

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Fig. 1. Linear production of glycerol by hepatocytes isolated from rainbow smelt (*Omerus mordax*) and incubated at 0.4°C. Each line represents an example of production from 1 individual. ○, fish acclimated to warm temperature (8°C) with 5 mM glucose in the medium (left y-axis); ●, fish acclimated to cold temperature (0.4 to −1.5°C) with 5 mM glucose in the medium (right y-axis); ▲, fish acclimated to 8°C with no additional substrate in medium (right y-axis).
converted to glycerol, this would result in a rate of \((215 - 17)/72\) = 5.5 \(\mu\)mol·g\(^{-1}\)·h\(^{-1}\), a value about twice as high as the observed rate of glycerol production.

Total GPase and GPase \(a\) activities were determined in the same population of cells as shown above for glycerol production. There was a significant increase in total GPase and GPase \(a\) activities when hepatocytes from warm fish were incubated at 0.4°C compared with preincubation activities (Fig. 4B), whereas an 8°C incubation showed a significant decrease in total GPase and GPase \(a\) activities (Fig. 4A). The directions of change match the patterns seen in glycerol production in hepatocytes isolated from warm fish at 0.4 and 8°C. There was no change in percent GPase \(a\) for either of these two groups. Individual variability in enzyme activity was much greater in the cold fish/0.4°C cells, and as a result, although average total GPase (7.02 vs. 11.75 \(\mu\)mol·min\(^{-1}\)·g\(^{-1}\)) and GPase \(a\) activities (5.73 vs. 11.58 \(\mu\)mol·min\(^{-1}\)·g\(^{-1}\)) were higher after incubation, they were not significantly different from preincubation values.

We next assessed whether adrenergic activation could work synergistically with low temperature to further increase glycerol production by isolated rainbow smelt hepatocytes (Table 2). The inclusion of 1 or 10 \(\mu\)M isoproterenol during the incubation period did not result in statistically significant changes in the production of glycerol relative to preparations without adrenergic stimulation. However, in warm cells incubated at 0.4°C, the average value of glycerol production decreased from 2.35 to 1.52 \(\mu\)mol·g\(^{-1}\)·h\(^{-1}\). In five experiments in which paired samples were available, the rate of glycerol production was decreased by 27 ± 8% with the addition of 1 \(\mu\)M isoproterenol. Isoproterenol did not change the activity patterns of total GPase, GPase \(a\) (data not shown), or percent GPase \(a\) (data not shown) relative to incubation without adrenergic stimulation. Total GPase activity in cells from both warm- and cold-acclimated fish was significantly higher in cells incubated at 0.4°C than in cells incubated at 8°C.

**Low-Temperature Incubation Redirects Glycogen-Derived Glucose to Glycerol**

In this series of experiments the hypothesis was tested that isolated hepatocytes produce glucose as well as glycerol.

<table>
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<th>Table 1. Rates of glycerol production by hepatocytes isolated from rainbow smelt (Osmerus mordax)</th>
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<td><strong>Incubation</strong></td>
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<tr>
<td><strong>Incubation</strong></td>
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<td>Glucose</td>
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Fish were acclimated to either warm (8°C) or cold temperature (0.4 to –1.5°C), and cells were incubated for 72 h at either 8 or 0.4°C. Medium contained 5 mM glucose, 5 mM aspartate, or no substrate. Rates were calculated by subtracting preincubation levels from the postincubation levels and dividing by 72 h. Glycerol production is in \(\mu\)mol·g\(^{-1}\)·h\(^{-1}\). Values are means ± SE; \(n=8\) for glucose (all conditions) and no substrate (0.4°C incubation) and \(n = 3–5\) for all other conditions. *,†,‡Different symbols indicate significant differences among different acclimation/incubation groups with glucose, aspartate, or no substrate in the medium.

**Fig. 2. Initial and final total (i.e., cellular plus extracellular) concentrations of glycerol in hepatocytes isolated from rainbow smelt and incubated for 72 h in medium without substrate or containing (in mM) 5 glucose, 5 alanine, 5 aspartate, or 1 pyruvate.**

**Fig. 3. Total glycerol concentration in hepatocytes isolated from rainbow smelt before incubation (solid bars) and 72 h after incubation (shaded bars).**

**Fig. 4. A:** Total glycerol production by isolated rainbow smelt hepatocytes incubated in media containing 5 mM glucose, 5 mM aspartate, or no substrate. Rates were calculated by subtracting preincubation levels from the postincubation levels and dividing by 72 h. Glycerol production is in \(\mu\)mol·g\(^{-1}\)·h\(^{-1}\). Values are means ± SE; \(n=8\) for glucose (all conditions) and no substrate (0.4°C incubation) and \(n = 3–5\) for all other conditions. *,†,‡Different symbols indicate significant differences among different acclimation/incubation groups with glucose, aspartate, or no substrate in the medium.
Hepatocytes were isolated from rainbow smelt acclimated to either warm (8°C) or cold temperature (0.4°C), and cells were incubated for 72 h at either 8 or 0.4°C. Hepatocytes were incubated for 72 h with no substrate in the medium and with or without addition of isoproterenol. Glycerol production is in μmol·g⁻¹·h⁻¹; GPase activity is in μmol·min⁻¹·g⁻¹·cells. Values are means ± SE; n = 6–7 for all conditions except for glycerol production with 1 μM isoproterenol in warm fish/0.4°C incubation, where n = 5, and glycerol production with 10 μM isoproterenol in warm fish/0.4°C incubation, where n = 4. * † ‡ Different symbols indicate significant differences among different acclimation/incubation groups with either no additions or isoproterenol in the medium.

Figure 5 compares glucose and glycerol levels after 72 h of incubation with and without 1 μM isoproterenol in the incubation medium. In all three groups, in the absence of isoproterenol, glucose levels increased significantly after 72 h compared with preincubation levels. This increase was highest in the warm fish/8°C cells (26.7 ± 7.8 μmol/g preincubation vs. 203.5 ± 71.6 μmol/g after 72-h incubation) and lowest in the cold fish/0.4°C cells (1.06 ± 0.13 μmol/g preincubation vs. 8.81 ± 3.02 μmol/g after 72-h incubation). Glycerol concentration in the warm fish/8°C cells was significantly decreased after 72 h. As anticipated, cells collected from warm fish and incubated at 0.4°C produced glycerol. Levels of glycerol in cells from warm fish following incubation were higher compared with levels in cells collected from cold fish and incubated at the same temperature.

The inclusion of 1 or 10 μM isoproterenol (10 μM data not shown) in the incubation medium had no impact on the final level of glucose accumulation relative to incubation without isoproterenol in any of the conditions and no impact on final glycerol level in cells from warm fish incubated at 8°C or cells from cold fish incubated at 0.4°C. However, 1 and 10 μM isoproterenol resulted in a significant decrease in the level of glycerol accumulation in cells from warm fish incubated at 0.4°C (from 79.0 ± 12.83 to 42.6 ± 6.35 and 40.1 ± 6.28 μmol/g with 1 and 10 μM isoproterenol, respectively). The only other impact of 10 μM isoproterenol was a significant increase in glucose production by cells from cold fish incubated at 0.4°C (from 1.06 ± 0.13 preincubation to 9.22 ± 2.97 μmol/g).

Rates of glucose and glycerol production/consumption are presented in Fig. 6 to allow comparisons across experimental preparations. Although not statistically significant, the average rate of glucose production in cells from warm-acclimated fish was lower at 0.4°C than in cells incubated at 8°C with or without isoproterenol in the medium. Hepatocytes isolated from cold fish and incubated at 0.4°C had the lowest rates of glucose production. When isoproterenol was included in the medium, a slight increase in rate of glucose production in the warm acclimation/0.4°C incubation condition was enough to make this group significantly different from the cold acclimation/0.4°C incubation group. In addition, the inclusion of isoproterenol resulted in a significant increase in the rate of glucose production from 0.74 ± 0.36 to 0.95 ± 0.36 μmol·g⁻¹·h⁻¹ in cells from warm fish incubated at 0.4°C. As in all previous experiments, cells from warm fish consumed glycerol when incubated at 8°C but produced glycerol when incubated at 0.4°C. In cells from warm fish incubated at 0.4°C, the inclusion of 1 μM isoproterenol resulted in a significant decrease in the rate of glycerol production (from 0.77 ± 0.17 to 0.27 ± 0.09 μmol·g⁻¹·h⁻¹). These data show that there is a redirection from glucose to glycerol production in hepatocytes isolated from warm rainbow smelt and incubated at low

**Table 2. Rates of glycerol production and total GPase activity in rainbow smelt hepatocytes**

<table>
<thead>
<tr>
<th></th>
<th>Warm Fish/8°C Incubation</th>
<th>Warm Fish/0.4°C Incubation</th>
<th>Cold Fish/0.4°C Incubation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycerol production</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No additions</td>
<td>-0.186 ± 0.035*</td>
<td>2.35 ± 0.45†</td>
<td>0.016 ± 0.024†</td>
</tr>
<tr>
<td>Isoproterenol (1 μM)</td>
<td>-0.193 ± 0.038*</td>
<td>1.52 ± 0.34†</td>
<td>-0.018 ± 0.019‡</td>
</tr>
<tr>
<td>Isoproterenol (10 μM)</td>
<td>-0.191 ± 0.037*</td>
<td>1.48 ± 0.30†</td>
<td>-0.027 ± 0.028‡</td>
</tr>
<tr>
<td>Total GPase</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No additions</td>
<td>5.06 ± 0.49*</td>
<td>13.44 ± 0.58†</td>
<td>11.75 ± 2.54†</td>
</tr>
<tr>
<td>Isoproterenol (1 μM)</td>
<td>4.65 ± 0.81*</td>
<td>13.52 ± 0.90†</td>
<td>11.53 ± 2.17†</td>
</tr>
<tr>
<td>Isoproterenol (10 μM)</td>
<td>4.65 ± 0.93*</td>
<td>13.41 ± 0.77†</td>
<td>11.00 ± 2.06†</td>
</tr>
</tbody>
</table>

**Fig. 4. Glycogen phosphorylase activities (total GPase and GPase a, left y-axis; %GPase a, right y-axis) in hepatocytes isolated from rainbow smelt before incubation (solid bars) and 72 h after incubation (shaded bars).**

A: hepatocytes isolated from fish held at 8°C and incubated at 0.4°C. B: hepatocytes isolated from fish held at 8°C and incubated at 0.4°C. C: hepatocytes isolated from fish held at 0.4°C and incubated at 0.4°C. All values are means ± SE; n = 6–7. *Significantly different from preincubation within the same acclimation/incubation temperature group.
Additional potential candidates of GPDH stimulation could by Glucocorticoid or PPAR/γ. Glycerol Production is Not Additionally Activated by 85 mg of protein (data not shown). When activities were expressed on the basis of wet weight was related to water content. The possibility that the difference in activity level based on wet weight was related to water content. HK and GK activities in whole liver were 0.13 ± 0.02 and 0.199 ± 0.04 μmol·min⁻¹·mg protein⁻¹, respectively. There was no significant difference between HK or GK activities in fresh liver and in cells isolated from different fish at the preincubation stage. Incubation of cells at either 0.4 or 8°C for 72 h did not alter HK or GK activities with respect to preincubation levels, and there was no difference in enzyme activities between cells incubated at 0.4 and 8°C. The same conclusions were reached when activities were expressed on the basis of milligrams of protein (data not shown).

Glycerol Production is Not Additionally Activated by Glucocorticoid or PPARα Agonists

In the next series of experiments we assessed whether additional potential candidates of GPDH stimulation could result in further increases in glycerol production than that due to low temperature alone. Cells isolated from warm-acclimated fish were incubated at 0.4°C for 72 h with 5 mM glucose in the incubation medium. As predicted, glycerol level was produced by cells with no additional agents (Fig. 7). Hydrocortisone (100 or 1,000 ng/ml), dexamethasone (100 or 1,000 ng/ml), or WY-14643 (50 μM) did not result in a further increase in glycerol accumulation.

**DISCUSSION**

The most important finding of this study is that in vitro low-temperature (0.4°C) challenge is sufficient to directly activate the glycerol production mechanism by liver cells isolated from rainbow smelt held at high temperature. Isolated hepatocytes incubated at 8°C did not produce glycerol. This was expected given that in vivo studies have shown that...
temperatures lower than 5°C are needed to activate glycerol synthesis (6, 12). Hepatocytes from cold-acclimated fish incubated at 0.4°C produced small amounts of glycerol when glucose was available but not under any other condition. This is consistent with low rates of glycerol production by fish in the plateau or glycerol decrease phase of the annual cycle. We emphasize that the current work focuses on the use of glycogen/glucose during a shift from warm to cold temperature. This study does not fully probe mechanisms for the maintenance of glucose production, may account for some variability in the glycero-genic potential of isolated liver cells. Furthermore, since sexually mature males and females were used in this study, variation in the glycerol production rates may be related to the reproductive cycle and lipid synthesis for gonad development. Regardless, there is absolute consistency within experiments in the activation of glycerol production by cells isolated from rainbow smelt at 8°C and incubated at 0.4°C. Additional extracellular signals related to adrenergic, glucocorticoid, or PPAR activation did not enhance glycerol production over that of low-temperature challenge alone. Thus the activation mechanisms for glycerol production are inherent to the hepatocytes.

In one experiment it was noted that when various substrates such as glucose, aspartate, and pyruvate were added to the hepatocyte medium, rates of glycerol production were greater than in the group with no substrate added. The enhanced production of glycerol when pyruvate or aspartate was available is in keeping with the concept that glycerol can be produced via the gluco- or glyceroenogenic pathway. Evidence for this includes 1) elevated levels of amino transferases (7), phosphoenolpyruvate carboxykinase (PEPCK) (12, 22), and PEPCK mRNA (13) in rainbow smelt liver either compared with other species or during the phase of rapid glycerol accumulation, and 2) the incorporation of injected radiolabeled and stable isotopes of pyruvate and amino acids into labeled glycerol (22, 23, 32).

Glycerol was produced by hepatocytes isolated from warm fish and incubated at 0.4°C even in the absence of exogenous substrate. Under these conditions, endogenous glycogen was utilized with most of the reserve being depleted after 72 h. This finding is consistent with whole animal studies in which glycogen levels in liver of rainbow smelt were decreased after several weeks of low-temperature exposure under a natural seasonal regime (29) and in days following an imposed decrease in temperature from 8 to −1.2°C (6). The glycogenolysis was associated with an increase in the total amount of measurable GPase activity and, more specifically, GPase a activity. The low-temperature challenge may have resulted in an increase in GPase gene expression; however, an alternative possibility exists. The activation of glycogen breakdown has been well studied in the wood frog, a species that produces glucose from massive glycogen stores as a primary cryoprotectant when exposed to ice crystallization. Cold exposure results in the conversion of an inactive, unmeasurable (by standard activity assays) b form into an active a form of GPase in the liver of this species (27). We cannot rule out the possibility that this occurs in liver of rainbow smelt as well. Glycogenolysis in the wood frog is under strong adrenergic control (27). This does not appear to be the case in rainbow smelt, at least with isolated liver cells. Exposure to isoproterenol (1 or 10 μM) in these experiments did not result in changes in GPase activity, led to only small increases in glucose production, and, most unexpectedly, decreases in glycerol production.

Glycogen utilization at 8°C did not result in glycerol production and, moreover, glycogen utilization in cells from warm fish incubated at 0.4°C exceeded that of glycerol accumulation. These findings motivated us to assess whether isolated liver cells were producing glucose, as occurs in many other species.

Table 3. Rates of glycerol production and GPDH, HK (low K_m), and GK (high K_m) activities in rainbow smelt hepatocytes

<table>
<thead>
<tr>
<th>Time</th>
<th>Glycerol</th>
<th>GPDH</th>
<th>HK</th>
<th>GK</th>
</tr>
</thead>
<tbody>
<tr>
<td>Preincubation</td>
<td>226±64.7*</td>
<td>0.20±0.04</td>
<td>0.25±0.04</td>
<td></td>
</tr>
<tr>
<td>0.4°C incubation</td>
<td>1.41±0.55*</td>
<td>1.41±0.38</td>
<td>0.28±0.05</td>
<td>0.27±0.02</td>
</tr>
<tr>
<td>8°C incubation</td>
<td>0.37±0.32</td>
<td>102±28.4</td>
<td>0.26±0.05</td>
<td>0.27±0.03</td>
</tr>
</tbody>
</table>

Hepatocytes were isolated from rainbow smelt acclimated to warm temperature (8°C) and incubated at either 0.4 or 8°C for 72 h. Glycerol production is in μmol·g\(^{-1}\)·h\(^{-1}\). Glycerol-3-phosphate dehydrogenase (GPDH), hexokinase (HK), and glucokinase (GK) enzyme activities are in μmol·min\(^{-1}\)·g\(^{-1}\). Values are means ± SE; n = 6. * Different symbols indicate significant differences among preincubation, 0.4°C incubation, and 8°C incubation groups.

**Fig. 7.** Total glycerol production in rainbow smelt hepatocytes isolated from warm-acclimated fish and incubated at 0.4°C for 72 h. All groups were incubated with 5 mM glucose in the medium. In addition, medium also included 100 or 1,000 ng/ml hydrocortisone (HC), 100 or 1,000 ng/ml dexamethasone (Dex), or 50 μM Wy-14643 (a peroxisome proliferator-activated receptor-α agonist). All values are means ± SE; n = 3.
of fish (16). Hepatocytes from warm-acclimated fish produced glucose at 8°C incubation temperature but not glycerol; however, incubation at 0.4°C resulted in not only glycerol production but equimolar release of glucose. An increase in GPase a activity following incubation at 0.4°C was sufficient to allow similar molar rates of glucose (at 8°C) compared with glucose plus glycerol release (at 0.4°C) despite the thermal difference. The switch from glucose production to a glucose plus glycerol production mode must involve control site(s) in addition to GPase. The finding that hepatocytes release glycerol at low temperature also raises the possibility that glucose is being exported to other tissues for extrahepatic glycerol production as opposed to the liver being the sole site of glycerol production for the animal.

A likely contributor to enhanced glycerol production at low temperature in the isolated cells is the enzyme GPDH. Whole animal studies involving enzyme activity, metabolite levels, and gene expression levels have revealed that enhanced glycerol production is associated with increased flux through GPDH (6, 12, 13). In the current study, a fourfold increase in rate of glycerol production was associated with maximal in vitro GPDH activities that were ~40% higher after 72 h of incubation at 0.4°C than at 8°C. GPDH activity in freshly isolated cells was higher than levels following incubation. We cannot account for this finding but suggest that there may be a transient increase in GPDH during the preparation of hepatocytes perhaps associated with a stress response. Regardless, various experiments have consistently shown that high activities of GPDH are associated with glycerol production. Given that the in vitro activity of GPDH is thousands of times higher than rates of glycerol production, it is unlikely that this is the only rate-controlling step in the metabolic pathway. As presented earlier, it may be that glycerol-3-phosphatase, which catalyzes the conversion of glycerol 3-phosphate to glycerol plus P_i, is a primary regulatory site (6). The maximal in vitro activity of glycerol-3-phosphatase at 20°C is 117 μmol·h^{-1}·g^{-1} (7). Assuming a Q_{10} of 2, this would equate to an activity of ~30 μmol·h^{-1}·g^{-1}, a value much closer to the highest rates of glycerol production observed here of 2.35 μmol·h^{-1}·g^{-1} than the activities of GPDH.

HK and GK levels were similar in isolated cells and in directly homogenized liver preparations, lending credibility to the concept that there was a transient stress-induced elevation in GPDH. The low-\(K_m\) HK and high-\(K_m\) GK activities are similar in rainbow smelt liver cells. This is different from the situation in rainbow trout, where GK is typically 10 to 15 times higher than HK activity. In the common carp (Cyprinus carpio), which has a high capacity to utilize dietary carbohydrate, HK activity is usually higher than GK activity (20). The data suggest that rainbow smelt, despite having a natural high protein diet and being more closely related to rainbow trout than common carp, has a liver metabolism designed for the glucose utilization, perhaps to fuel essential glycerol production at low temperatures. The activities of HK and GK were not altered by incubation at either 0.4 or 8°C. HK kinetics have been studied in the glycerol-producing larvae of the goldenrod gall moth (Epiblema scudderiana). At low temperature the enzyme has a higher affinity for glucose and ATP and reduced product inhibition by G6P and ADP than at high temperature (18). If this were to occur in rainbow smelt liver as well, it could contribute to the utilization of extracellular glucose for glycerol production and might be one of the factors involved in higher rates of glycerol formation in cells incubated at 0.4°C than at 8°C, where glucose was available in the medium (e.g., Tables 1 and 3). The kinetic properties of HK also may be critical in the switch from glucose release at high temperature to a mixed glucose and glycerol release at low temperature if the enzyme serves to salvage glucose released from glycogenolysis and redirect glucose into the glycolytic pathway. Any downstream regulation between fructose-6-phosphate and glycerol could decrease the level of G6P and thus relieve any potential product inhibition on HK. This scenario remains speculative until detailed analysis of metabolite levels and the kinetic properties of HK are achieved.

One feature of the study that remains unresolved is an apparent inhibitory effect of isoproterenol on glycerol production as suggested in the experiment reported in Table 2 and reported as a significant reduction of 65% in Fig. 6. Isoproterenol inhibits pyruvate kinase (PK) in rainbow trout liver (33). It may be that a requirement for glycerol production is an ongoing glycolysis to provide cytosolic NADH for GPDH activity. An inhibition of glycolysis through PK activity would reduce flux though GPDH and in turn could impede glycerol production. In the whole animal context, this may be that adrenergic control is involved in the decrease in glycerol production as the season progresses and not the increase in glycerol accumulation in the fall.

**Perspectives and Significance**

Rainbow smelt accumulate high levels of glycerol throughout the body as an antifreeze mechanism. At the whole animal level, glycerol production is triggered by a decrease in water temperature to 3–5°C (6). In this study we have shown that a low-temperature signal alone is sufficient to activate the glycerol production mechanism in liver cells, since additional putative signals have no further stimulatory impact. Glycerol production in isolated hepatocytes may be fueled by a variety of substrates such as glucose and amino acids; however, cells make glycerol in the absence of substrate from glycolgen stores. A decrease in temperature results in a shift from glucose release to a mix of glucose and glycerol release. Glycogenolysis is activated in part through an increase in the total activity of GPase, and high levels of a low-\(K_m\) HK may serve to redirect glycogen-derived glucose to glycolysis as opposed to release from the cells. Increases in GPDH activity are associated with elevated levels of glycerol production, but strong metabolic control most likely exists at other sites as well. An understanding of glycerol production mechanisms has relevance beyond the current model system of rainbow smelt per se. The process of glyceroneogenesis leading to glycerol-3-phosphate in mammalian liver is an active area of research, because it is essential to the triglyceride/fatty acid cycle and is a target for treatment of type 2 diabetes (10, 24, 30). In addition, activity of a putative glycerol-3-phosphatase has been suggested in oxygen-limited rat heart and brain, although neither the protein nor the transcript have been identified (3, 19). The current work with rainbow smelt presents an ideal model system to study the control mechanisms of glyceroenogenesis and the final step in glycerol production. Also, along with earlier studies, we have shown that GPDH is an unanticipated modulated step in the process.
ACKNOWLEDGMENTS

We thank Connie Short for technical assistance and the Field Services Unit of the Ocean Sciences Centre for the collection of specimens. We thank Dr. Kelly Soanes, National Research Council Canada (NRC) Institute for Marine Biosciences, for reviewing this manuscript. This is NRC publication 2007-42743.

GRANTS

This work was supported by a Natural Sciences and Engineering Research Council of Canada Discovery Grant (W. R. Driedzic), the Newfoundland and Labrador Department of Entrepreneurship, Trade and Rural Development (W. R. Driedzic), and the Canadian Institutes of Health Research through the Regional Partnership Program (W. R. Driedzic and K. V. Ewart). W. R. Driedzic holds the Canada Research Chair in Marine Bioscience.

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