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RESEARCH ARTICLE

Glucose uptake and metabolism by red blood cells from fish with different extracellular glucose levels

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SUMMARY

The aim of the present study was to assess whether mechanisms of glucose trafficking by red blood cells (RBCs) relate to species-specific extracellular glucose levels. Atlantic cod (*Gadus morhua*), Atlantic salmon (*Salmo salar*), cunner (*Tautogolabrus adspersus*) and short-horned sculpin (*Myoxocephalus scorpius*) had plasma glucose levels of 4, 4.1, 1.95 and 0.73 mmol l^{-1}, respectively. Glucose uptake by isolated RBCs was measured by the initial incorporation of [6-^{14}C]-glucose and steady-state glucose metabolism was determined by the production of $^{3}H_2O$ from [2-^{3}H]-glucose. Saturation kinetics of glucose uptake and inhibition of both glucose uptake and metabolism by cytochalasin B and phloretin revealed that Atlantic cod, cunner and sculpin RBCs all had a facilitated transport component to glucose trafficking. RBCs from Atlantic salmon showed a linear relationship between glucose uptake and extracellular glucose level, but exhibited clear inhibition of glucose metabolism by cytochalasin B and phloretin, suggesting a component of facilitated glucose transport that is more elusive to detect. The production of $^{3}H_2O$ was linear for at least 6 h and as such presents a rigorous approach to measuring glycolytic rate. Steady-state rates of glucose metabolism were achieved at extracellular levels of approximately 1 mmol l^{-1} glucose for RBCs from all species, showing that within-species normal extracellular glucose level is not a primary determinant of the basal level of glycolysis. At physiological levels of extracellular glucose, the ratio of initial glucose uptake to glucose metabolism was 1.5 to 4 for all RBCs, suggesting that there is scope to increase metabolic rate without alteration of the basal glucose uptake capacity.

Key words: glucose transporter, [2-^{3}H]-glucose, cytochalsin B, phloretin, Atlantic cod, *Gadus morhua*, Atlantic salmon, *Salmo salar*, cunner, *Tautogolabrus adspersus*, short-horned sculpin, *Myoxocephalus scorpius*.

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INTRODUCTION

There is substantial variability in blood glucose concentration within and amongst fish, with some species having levels lower than 1 mmol l^{-1} and others with levels in excess of 10 mmol l^{-1} (Chavin and Young, 1970; Polakof et al., 2011). This is in contrast to mammals, where blood glucose level is typically maintained close to 7 mmol l^{-1} (Polakof et al., 2011). Glucose uptake is controlled by the concentration gradient of extracellular to intracellular glucose and the capacity of glucose to move across the membrane by either simple or facilitated diffusion. The level of intracellular glucose and subsequent metabolism is determined primarily by the activity of hexokinase (Wasserman et al., 2011). A first approximation calculation based on values for whole blood glucose, plasma glucose and hematocrit presented in Rodrigues et al. (Rodrigues et al., 1999) for seven different fish species from polar to tropical environments shows that generally the higher the level of blood glucose the steeper the diffusion gradient from the plasma space to the intracellular space of red blood cells (RBCs), and that the concentration of glucose in the RBC is on average only ~3% of the concentration in the plasma. There is an extensive literature dealing with glucose trafficking in fish RBCs but as of yet a coherent pattern is not evident. Here we question whether species-specific differences in blood glucose concentration relate to the potential for glucose uptake (i.e. passage across the cell membrane) and/or steady-state glycolysis in fish RBCs.

The capacity for glucose uptake by fish RBCs has been assessed by the short-term accumulation of radiolabelled metabolites. RBCs from Pacific hagfish (*Eptatretus stouti*) (Ingermann et al., 1984; Young et al., 1994), lamprey (*Lampetra fluviatilis*) (Tiihonen and Nikinmaa, 1991a), common eel (*Anguilla japonica*) (Tse and Young, 1990) and American eel (*Anguilla rostrata*) (Soengas and Moon, 1995) exhibit facilitated glucose transport. This contention is supported by saturation kinetics of [^{14}C]-OMG (3-*O*-methyl-D-glucose; a non-metabolized glucose analog) and/or D-[^{14}C]-glucose, as well as inhibition of uptake by cytochlasin B and/or phloretin. The concept of facilitated transport is further strengthened by the presence of a glucose transporter 1 (GLUT1)-like transporter protein in RBCs of Pacific hagfish (Young et al., 1994), the presence of GLUT1 and GLUT3 mRNAs in Atlantic cod RBCs (J. R. Hall and W.R.D., in preparation) and GLUT1 transcripts at low levels in blood of rainbow trout (*Onchorynchus mykiss*) (Teerijoki et al., 2000). In contrast, studies involving a number of other species have concluded that glucose uptake is primarily by passive diffusion. The evidence for this is, foremost, a linear relationship between [^{14}C]-OMG or [^{14}C]-glucose incorporation and the concentration of extracellular substrate in RBCs from five species of Amazonian fish (Kim and Isaacks, 1978), carp (*Cyprinus carpio*) (Tiihonen and Nikinmaa, 1991b; Tiihonen et al., 1995) and brown trout (*Salmo trutta*) (Pesquero et al., 1992), and secondly a lack of or only modest inhibition by cytochlasin B or phloretin on [^{14}C]-OMG and/or D-[^{14}C]-glucose uptake in paddyfield eel (*Monopterus albus*), rainbow trout (Tse and Young, 1990) and carp (Tiihonen and Nikinmaa, 1991b). It appears that the presence or absence of facilitated glucose

transport in fish RBCs is species specific; alternatively the experimental conditions or approaches used to assess glucose uptake may have lacked the necessary power of resolution.

Insight into glucose metabolism by fish RBCs is revealed by measurements of oxygen consumption and lactate production under normoxic conditions. Oxygen consumption has been reported for RBCs from numerous species [e.g. rainbow trout (Ferguson et al., 1989; Walsh et al., 1990; Sephton and Driedzic, 1994a; Phillips et al., 2000); Atlantic salmon, *Salmo salar* (Ferguson and Boutilier 1988); brown trout (Pesquero et al., 1992); sea raven, *Hemitripterus americanus* (Sephton and Driedzic, 1991; Sephton and Driedzic, 1994a); and American eel (Soengas and Moon, 1995)], and is considered to be common to fish RBCs. Under normoxic conditions, RBCs from sea raven (Sephton and Driedzic, 1991; Sephton and Driedzic, 1994a) and three species of salmonids [rainbow trout (Ferguson et al., 1989; Walsh et al., 1990; Sephton and Driedzic, 1994a; Phillips et al., 2000); brown trout (Pesquero et al., 1992); and Atlantic salmon (Ferguson and Boutilier, 1988)] produce only minimal levels of lactate relative to calculated aerobic ATP production based on oxygen consumption. These studies rule out a substantial anaerobic glucose metabolism. Efforts have been made to assess the rate of aerobic glucose metabolism from the rate of $^{14}CO_2$ production from [^{14}C]-glucose [rainbow trout (Walsh et al., 1990; Sephton and Driedzic, 1994a); sea raven (Sephton and Driedzic, 1991; Sephton and Driedzic, 1994a)]. In these experiments, glucose metabolism calculated from collection of $^{14}CO_2$ and specific activity of extracellular glucose is much lower than oxygen consumption. The most likely explanation for the mismatch is that the radiolabel equilibrates with various intracellular metabolites, so the specific activity of the sites that actually produce $^{14}CO_2$ (e.g. specific activity of pyruvate for production of CO_2 *via* pyruvate dehydrogenase) is much lower than extracellular [^{14}C]-glucose specific activity and therefore rates of glucose metabolism based on extracellular glucose specific activity are underestimates. This interpretation is supported by the finding that in sea raven and rainbow trout RBCs incubated for 2 h with [^{14}C]-glucose, most of the radioactivity remains in the intracellular acid-soluble pool (Sephton and Driedzic, 1994a) and that radioactivity in the acid-soluble fraction increases in a linear fashion with respect to time [rainbow trout (Sephton and Driedzic, 1994a)]. Metabolic fuel preference by RBCs has been assessed by incubating cells in media containing various [^{14}C]-labeled substrates, including glucose, pyruvate, lactate, various amino acids and adenosine [carp (Tiihonen and Nikinmaa, 1991b); American eel (Soengas and Moon, 1995); rainbow trout (Walsh et al., 1990)]. Again, without knowing the specific activity of the substrates that release $^{14}CO_2$, it is not possible to resolve rates of oxidation from these data. It is clear that RBCs from fish have the capability to metabolise a range of substrates and that all fish reported to date can oxidize glucose to CO_2; however, an alternative approach to measuring rates of glucose metabolism is required.

How the matrix of physiological levels of blood glucose, the maximal rate of glucose uptake and the presence or absence of a facilitated transport process are interconnected to the steady-state rate of glucose metabolism is unknown. Here we assess whether glucose uptake and metabolism by RBCs are proportional to species-specific extracellular glucose. RBCs were obtained from species that were expected to have low [short-horned sculpin, *Myoxocephalus scorpius* (Linnaeus 1758)], intermediate [cunner, *Tautogolabrus adspersus* (Walbaum 1792)] and high (Atlantic cod, *Gadus morhua* Linnaeus 1758, and Atlantic salmon, *Salmo salar* Linnaeus 1758) levels of blood glucose. The latter two species with similar levels of blood glucose were included because the available information suggests that RBCs from Atlantic cod have facilitated transport mechanisms, whereas salmonid RBCs do not. Studies followed the classic pattern of time course evaluation to determine a period of linearity of uptake/metabolism, changes in extracellular concentration of glucose to assess whether uptake/metabolism saturated, and inclusion of cytochalasin B and phloretin, two well-recognized inhibitors of facilitated glucose transport. Glucose uptake was measured by tracking the incorporation of D-[^{14}C]-glucose into the acid-soluble pool as in previous reports (Tse and Young, 1990; Young et al., 1994; Sephton and Driedzic, 1994a; Sephton and Driedzic, 1994b). This approach captures all of the radiolabel that enters the cell and may remain as glucose or have alternative fates. It is considered quite suitable for studies with fish RBCs because over the time course of these experiments $^{14}CO_2$ production from labelled glucose is only ~1% of what remains in the intracellular pool (Sephton and Driedzic, 1994a). Steady-state glucose metabolism was assessed by tracking the release of ^3H_2O from [2-^{3}H]-glucose as described for mammalian RBCs (Hutton, 1972). The method is based upon the release of tritiated water when [2-^{3}H]-glucose 6-phosphate is converted to fructose 6-phosphate by phosphohexose isomerase, thus giving a measure of glycolysis. The procedure may be used in cells such as RBCs that have low rates of pentose phosphate pathway activity (Hutton, 1972). Our major novel findings are that steady-state glucose metabolism saturates at very low levels of extracellular glucose and that at physiological levels of extracellular glucose, the ratio of initial uptake/basal metabolism is approximately 2–4 for all species tested.

MATERIALS AND METHODS

Animal collection and sampling

Atlantic cod, Atlantic salmon, cunner and short-horned sculpin were maintained in running seawater at a temperature of 8–10°C and kept on a natural photoperiod with fluorescent lights set by an outdoor photocell. Atlantic salmon were hatchery reared by Cold Ocean Salmon (Daniel's Harbour, NL, Canada) and transferred to the Ocean Sciences Centre, Memorial University of Newfoundland (St John's, Canada), in September 2008. Specimens from the same population were used throughout the study. Atlantic cod were hatchery reared at the Ocean Sciences Centre in 2009 and 2010. Atlantic salmon and Atlantic cod received commercial pellets every day. Cunner and short-horned sculpin were collected locally as required by the field services unit (Ocean Sciences Centre) during the summer period, and were fed to satiation weekly with chopped herring. In some cases, blood was obtained from the same animal for more than one experiment, with a minimal holding time of 2 weeks between sampling dates. Body masses ranged from 800 to 2500 g, 1000 to 1500 g, 150 to 250 g and 500 to 800 g for Atlantic salmon, Atlantic cod, cunner and short-horned sculpin, respectively. Animal protocols were approved by the Animal Care Committee at the Memorial University of Newfoundland.

Blood preparation

RBCs were prepared based on the method by Moon and Walsh (Moon and Walsh, 1994). Approximately 1.25 ml blood was removed from the caudal vessel with a heparinized syringe and delivered to a preweighed Eppendorf centrifuge tube. Blood was immediately centrifuged at 1500 ***g*** for 5 min. Plasma was collected and frozen for future glucose analysis. The RBC pellet was washed three times with TES buffer containing (in mmol l^{-1}): 182 NaCl, 5 KCl, 1.99 $MgSO_4$, 2.3 $CaCl_2$, 7.33 TES base and 2.58 TES acid (pH 7.8 at 10°C). During the first two washes, the RBCs were centrifuged at 1500 ***g*** for 5 min;

following the third wash, the cells were centrifuged at 5000 ***g*** for 5 min. If necessary, cells from two or three fish were combined at this point and resuspended to ~20% hematocrit in TES with or without glucose depending on the experiment.

Plasma glucose levels

Plasma was assayed as described by Clow et al. (Clow et al., 2004) with the exception that absorbance was determined with a DTX 880 microplate reader (Beckman Coulter, Mississauga, ON, Canada).

Glucose uptake following the incorporation of [6-^{14}C]-glucose

The first series of experiments assessed rates of glucose update by measuring the incorporation of [6-^{14}C]-glucose into the acid-soluble pool. All incubations were conducted in duplicate in 16×100 mm glass tubes containing: 50 µl TES containing [6-^{14}C]-glucose [ethanol free; 3.7 kBq; Perkin Elmer, Woodbridge, Canada], 50 µl of TES with or without a known concentration of glucose, and 100 µl of RBC suspension. Preliminary studies revealed that recoverable radioisotopes in the RBC pellet were higher and more consistent with [6-^{14}C]-glucose than with [1-^{14}C]-glucose. In initial time course experiments, RBCs were incubated in medium containing 5 mmol l^{-1} glucose for periods of up to 200 min. In all cases, RBCs were incubated within 1 h from time of blood collection. All experiments were performed at 8°C.

In glucose concentration experiments, RBCs were sampled immediately after the mixing of cells with incubation medium (considered to be time zero) and thereafter following 10 min of incubation in the case of Atlantic salmon, and initially 30 min of incubation for all other species. Glucose uptake was assessed at extracellular levels of 0.078, 0.1, 0.156, 0.312, 0.625, 1, 1.25, 2, 2.5, 5, 7.5, 10, 15 and 20 mmol l^{-1} glucose in the final incubation medium. A further experiment was conducted with Atlantic cod with a 2.5 min incubation period and extracellular glucose levels of 1.25, 2.5, 5, 7.5, 10, 12.5, 15, 17.5 and 20 mmol l^{-1}. In experiments with Atlantic salmon, cunner and short-horned sculpin, cell preparation and incubation were initiated upon blood removal. Isolated RBCs from Atlantic cod were held at 8°C for 18 h prior to incubation. This approach was taken as preliminary experiments showed that RBCs from Atlantic cod remained quiescent with respect to glucose uptake for many hours but showed substantial increases in glucose uptake after being held overnight. For all other species there was no difference in glucose uptake by RBCs utilized immediately after collection or after being held overnight.

In order to gain further insight as to whether glucose uptake was by passive or facilitated transport, the next experiment involved two inhibitors: phloretin, a general inhibitor of facilitative transport, and cytochalasin B, which impairs facilitated glucose transport into cells by binding to glucose transporter proteins (Silverman, 1991). RBCs were incubated with either 0.3 mmol l^{-1} phloretin or 25 µmol l^{-1} cytochalasin B for 30 min prior to the addition of glucose. Because both inhibitors were dissolved in DMSO, RBCs were also incubated with 0.33% DMSO. RBCs were incubated with either 0.5 or 5 mmol l^{-1} glucose for 30 min, except for Atlantic salmon RBCs, which were incubated for 10 min.

After the incubations, 150 µl of the RBC suspension was removed and layered over 400 µl *N*-dibutylphthalate (DBP) (Moon and Walsh, 1994) in a preweighed Eppendorf tube. Samples were immediately centrifuged at 12,000 ***g*** for 30 s. DBP, which has a density of 1.04 g ml^{-1}, was used to quickly separate the RBCs from the suspension medium using fast centrifugation. A 20 µl aliquot of the supernatant was removed for the determination of extracellular glucose specific activity. The remaining supernatant and DBP layer were removed. Nine millilitres of perchloric acid per gram of pelleted RBC were added to the tube and allowed to stand for 30 min. The samples were centrifuged at 12,000 ***g*** for 10 min and an aliquot of the perchloric acid was removed for counting in EcoLume (MP Biomedicals, Montreal, Canada). Preliminary experiments using ^{3}H and ^{14}C mannitol confirmed that there was only a minimal amount (0.5–1.4%) of extracellular fluid associated with the pelleted RBCs.

In the [^{14}C]-glucose time course experiments, the initial time point is considered to be time zero, but in practice it was closer to 20–30 s by the time the [^{14}C]-glucose and RBCs were mixed, layered on the oil and centrifugation was initiated. Counts in this fraction were subtracted from the remainder of the time points. In the [^{14}C]-glucose concentration experiments, a zero time point was run with every glucose level. Again, the zero value was subtracted from the counts determined after the incubation period. Time zero correction was essential as background counts in some cases represented 80–90% of the radioactivity recovered following incubation.

Glucose metabolism following the production of $^{3}H_2O$

In a second series of experiments, the steady-state rate of glucose metabolism was determined. In these experiments, RBCs were held ~18 h prior to incubations. Cells were collected and prepared as above; however, [2-^{3}H]-glucose [7.4 kBq; American Radiolabeled Chemicals, Burnaby, Canada] was used in the incubation medium instead of [6-^{14}C]-glucose. The metabolism of glucose was determined by measuring the rate of $^{3}H_2O$ production that is formed from [2-^{3}H]-glucose *via* water exchange in the reaction catalyzed by phosphoglucose isomerase (Hutton, 1972). Time course experiments were performed over a period of 1–6 h with medium containing 5 mmol l^{-1} glucose. The relationship between extracellular glucose level and glucose metabolism was determined using concentrations as above, but the incubation time was 5 h for all species. Incubations with inhibitors were performed as described above except with a 5 h time point.

At the end of the incubation, cells were quickly transferred from the glass tubes to preweighed Eppendorf tubes and centrifuged at 12,000 ***g*** for 30 s. The supernatant was collected, transferred to another tube and put into liquid nitrogen. The [2-^{3}H]-glucose and the $^{3}H_2O$ were separated using chromatography as described by Broderick et al. (Broderick et al., 1992). Dowex 1X4 chloride was converted to the borate form by adding 0.4 mol l^{-1} borate (potassium tetraborate tetrahydrate) and left stirring overnight. The next day, the resin was rinsed with several volumes of distilled water until the pH was between 7 and 8. Separation of $^{3}H_2O$ and [^{3}H]-glucose was achieved by adding 100 µl of sample supernatant to ~1 ml of resin in polystyrene columns. Four 1 ml water fractions containing the $^{3}H_2O$ produced by the cells were collected in scintillation vials and counted. The labelled glucose remained on the column. Time zero values were subtracted from values obtained after the incubation periods as described for the [^{14}C]-glucose experiments above. In this case, background counts were always minimal.

Data analysis

Values are expressed as means ± s.e.m. Comparisons amongst species were made using a one-way ANOVA with a Tukey's *post hoc* test. Significant differences involving more than two conditions within a species were assessed with one-way repeated measures ANOVA with Dunnett's *post hoc* test. Maximal enzyme velocity (V_{max}) and the Michaelis–Menton constant (K_m) were determined by non-linear regression. In all cases $P<0.05$ was considered to be significant.

Fig. 1. Plasma glucose levels in Atlantic cod (*N*=36), Atlantic salmon (*N*=27), cunner (*N*=28) and short-horned sculpin (*N*=27). Values that do not share a common letter are significantly different.

RESULTS

Plasma glucose

Plasma glucose levels in Atlantic cod and Atlantic salmon were similar and significantly higher than both cunner and short-horned sculpin, and the level in cunner was significantly higher than in short-horned sculpin (Fig. 1). There was a 5.6-fold difference in plasma glucose between the two species with highest levels and short-horned sculpin.

Initial rate of glucose uptake

Fig. 2 shows the time course of incorporation of [6-^{14}C]-glucose into the acid-soluble pool of RBCs with 5 mmol l^{-1} glucose in the extracellular medium. Incorporation is considered to be linear for 40 and 75 min for RBCs from cunner and short-horned sculpin, respectively. For RBCs from Atlantic salmon, linearity of glucose uptake tails off after 30 min. The situation in Atlantic cod is more difficult to interpret. There was linearity of glucose uptake between 5 and 60 min but a sharp increase between time zero and 5 min. These experiments served as the foundation for setting the time of incubation for glucose concentration experiments to 10 min for RBCs from Atlantic salmon, 30 min for cunner and short-horned sculpin, and both 30 min and 2.5 min for Atlantic cod.

The relationship between the initial rate of incorporation of [6-^{14}C]-glucose into the acid-soluble pool of RBCs and extracellular glucose concentration is presented in Fig. 3. The rate of glucose incorporation became saturated in cunner (V_{max}=8.9 nmol g^{-1} min^{-1}, K_m=7.8 mmol l^{-1}) and short-horned sculpin (V_{max}=9.4 nmol g^{-1} min^{-1}, K_m=2.1 mmol l^{-1}).

For Atlantic cod RBCs, incubated for 30 min, the incorporation of glucose continued to increase in a linear fashion up to 10 mmol l^{-1} but decreased at 20 mmol l^{-1} extracellular glucose. One explanation for the apparent decrease in rate at 20 mmol l^{-1} glucose is that at this concentration uptake is not linear for the 30 min time period selected in these experiments but rather decreases over time. This would lead to an underestimate of glucose uptake when normalized per minute. In order to address this concern, RBCs were incubated for 2.5 min with an emphasis on higher extracellular concentrations of glucose (Fig. 3A, inset). Under this protocol, uptake became saturated at ~10 mmol l^{-1} extracellular glucose. The combined data sets with the exclusion of the aberrant value at 20 mmol l^{-1} glucose yielded a V_{max} of 13.5 nmol g^{-1} min^{-1} and a K_m of 4.7 mmol l^{-1}.

For Atlantic salmon RBCs, the incorporation of glucose continued to increase in a linear fashion up to the 20 mmol l^{-1} glucose, the highest level tested.

The initial glucose uptake rates at close to physiological levels of extracellular glucose are presented in Fig. 4A. There was no

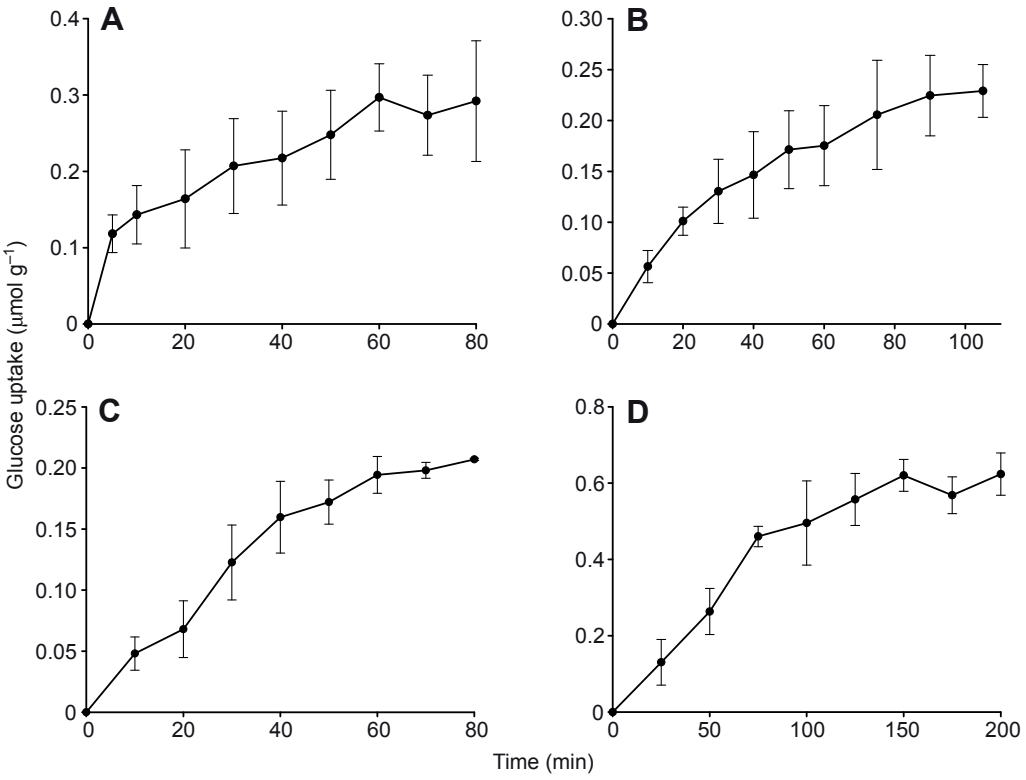

Fig. 2. Time course of incorporation of [6-^{14}C]-glucose into the acid-soluble pool of red blood cells (RBCs) from (A) Atlantic cod, (B) Atlantic salmon, (C) cunner and (D) short-horned sculpin. RBCs were incubated with 5 mmol l^{-1} glucose in the extracellular fluid at a temperature of 8°C. *N*=3 for Atlantic cod, cunner and short-horned sculpin; *N*=4 for Atlantic salmon. Data are means ± s.e.m.

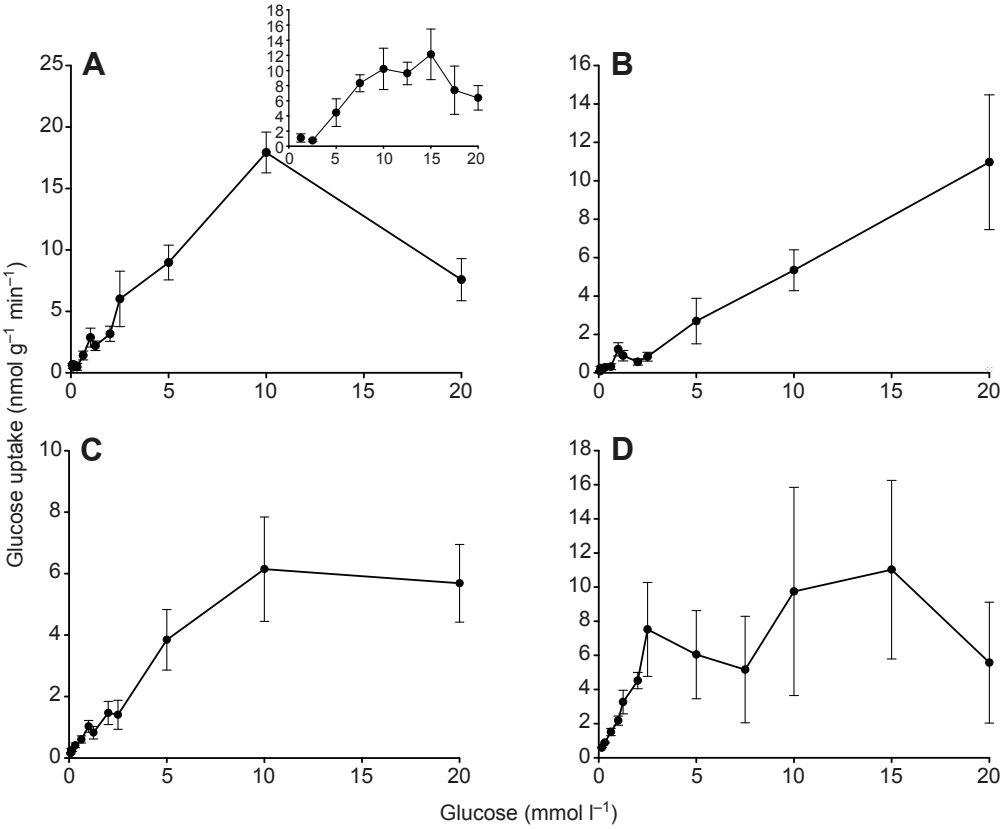

Fig. 3. Relationship between glucose uptake and extracellular glucose concentration in RBCs from (A) Atlantic cod, (B) Atlantic salmon, (C) cunner and (D) short-horned sculpin. Time of incubation was as follows: Atlantic cod (30 min main panel; 2.5 min inset); Atlantic salmon (10 min); and cunner and short-horned sculpin (30 min). Incubation temperature was 8°C. *N*=4 for Atlantic salmon; *N*=3 for all other species. Data are means ± s.e.m.

significant difference in glucose uptake amongst short-horned sculpin, cunner and Atlantic salmon; however, the rate of glucose uptake by Atlantic cod RBCs was significantly higher than the rate for either cunner or short-horned sculpin RBCs.

Glucose metabolism

The technique of tracking ^3H_2O production from [2-^{3}H]-glucose provides a measure of carbon flux through the glycolytic pathway. The time course of glucose metabolism at an extracellular glucose concentration of 5 mmol l^{-1} is presented in Fig. 5. The production of ^3H_2O is linear for RBCs from all species for at least 360 min. The curves do not saturate as the technique reflects steady-state and sustainable rates of metabolism.

The relationship between the rate of metabolism and extracellular glucose concentration is shown in Fig. 6. Maximal rates of metabolism were reached for RBCs from every species by ~1 mmol l^{-1} extracellular glucose and increased very little thereafter. Rates of glucose metabolism at approximately physiological levels of extracellular glucose are presented in Fig. 4B. Glucose metabolism was significantly higher by RBCs from Atlantic cod than either RBCs from cunner or short-horned sculpin, with rates of metabolism being similar in the latter two species. The rate of glucose metabolism by RBCs from Atlantic salmon was intermediate between Atlantic cod and both cunner and short-horned sculpin. More specifically, rates of glucose metabolism by RBCs were 2.58 nmol g^{-1} min^{-1} for Atlantic cod (incubated with 5 mmol l^{-1} glucose in the medium), 1.36 nmol g^{-1} min^{-1} for Atlantic salmon (with 5 mmol l^{-1} extracellular glucose), 0.34 nmol g^{-1} min^{-1} for cunner (with 2.5 mmol l^{-1} extracellular glucose) and 0.52 nmol g^{-1} min^{-1} for short-horned sculpin (with 1 and 0.5 mmol l^{-1} glucose in the medium).

Uptake relative to metabolism

The ratio of the initial rate of glucose uptake to the rate of steady-state metabolism is presented in Fig. 4C. This analysis provides insight into whether uptake or metabolism is limiting to the overall utilization of glucose. Values greater than 1 imply that the capacity to take up glucose exceeds the basal metabolic rate. At low levels of extracellular glucose, RBCs from cunner and short-horned sculpin have excess uptake capacity relative to rates of glucose metabolism as evidenced by ratios that range from 2 to 4. Glucose uptake and glucose metabolism appear to be closely matched in RBCs from Atlantic cod and Atlantic salmon at low levels of extracellular glucose; however, at extracellular glucose levels of 5 mmol l^{-1}, the ratio of uptake/metabolism increases to 2 to 3 for these two species.

Inhibition experiments

Fig. 7 shows the impact of the transport inhibitors phloretin and cytochalsin B on glucose uptake by RBCs from Atlantic cod, cunner and short-horned sculpin at extracellular glucose levels of 0.5 and 5 mmol l^{-1}. Unfortunately, for unknown reasons, it was not possible to obtain reproducible data for RBCs from Atlantic salmon. In the absence of inhibitors, glucose uptake rates were consistent with results of the glucose concentration experiment presented in Fig. 3 and in no case did the inclusion of DMSO in the medium significantly alter glucose uptake. At an extracellular level of 0.5 mmol l^{-1} glucose, both phloretin and cytochalasin B significantly inhibited glucose uptake by RBCs from all three species. The one exception to this finding was with cunner RBCs treated with cytochalasin B, where although there was a decrease in the average value of glucose uptake with respect to controls, the difference was not statistically significant. Other than this anomaly, the impact of

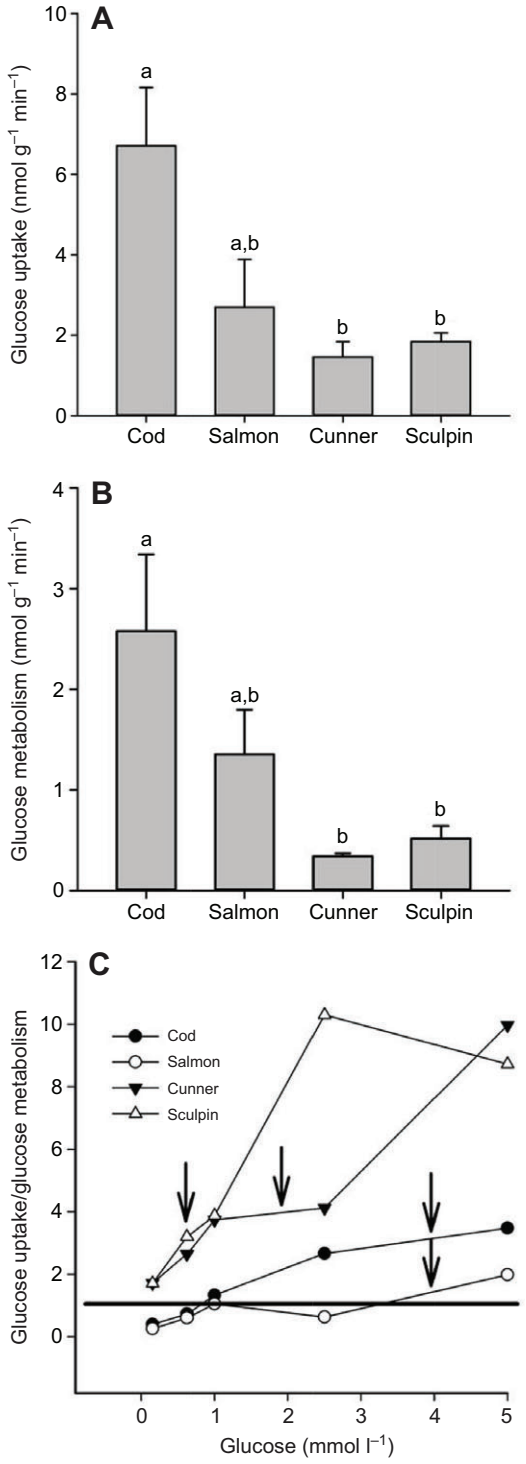

Fig. 4. Initial rate of glucose uptake and steady-state glucose metabolism by RBCs at extracellular glucose concentrations approximating species-specific physiological concentrations of plasma glucose. (A) Glucose uptake. Data are extracted from Fig. 3 as follows: Atlantic cod at 5 mmol l^{-1} extracellular glucose (based on the average of the two experiments); Atlantic salmon at 5 mmol l^{-1} glucose; cunner at 2.5 mmol l^{-1} glucose; short-horned sculpin at 0.625 and 1 mmol l^{-1} glucose. (B) Glucose metabolism. Data are extracted from Fig. 6 as follows: Atlantic cod and Atlantic salmon at 5 mmol l^{-1} glucose; cunner at 2.5 mmol l^{-1} glucose; and short-horned sculpin at 0.5 and 1 mmol l^{-1} glucose. Values that do not share a common letter are significantly different. (C) Ratio of initial rates of glucose uptake to rates of glucose metabolism *versus* extracellular glucose concentration. Arrows indicate the physiological concentration of glucose.

inhibition of glucose uptake was considerable at ~80% of the control value for all situations. The impact of phloretin and cytochalsin B on glucose uptake with 5 mmol l^{-1} glucose in the medium was less substantive. In all situations the average value for glucose uptake in the presence of putative inhibitors was less than the respective controls, suggesting an impairment of uptake, but values were not significantly different within any species tested.

The impact of the transport inhibitors phloretin and cytochalsin B on glucose metabolism (i.e. ^3H_2O production from [2-^{3}H]-glucose) by RBCs at extracellular glucose levels of 0.5 and 5 mmol l^{-1} is shown in Fig. 8. Rates of metabolism were consistent with levels noted in Fig. 6 and in no case did the inclusion of DMSO have an effect. In all cases, phoretin and cytochalasin B resulted in a significant decrease in glucose metabolism. This occurred for RBCs from all species and at both levels of extracellular glucose. The degree of inhibition of glucose metabolism was substantial. At 0.5 mmol l^{-1} extracellular glucose, metabolism was generally inhibited by 88±1.9%, and at 5 mmol l^{-1} extracellular glucose, metabolism was inhibited by 74±2.8%.

DISCUSSION

Blood glucose

As anticipated, the experimental species selected for study showed differences in plasma glucose, with Atlantic cod and Atlantic salmon having the highest levels (~4 mmol l^{-1}), followed by cunner (~2 mmol l^{-1}) and short-horned sculpin (<1 mmol l^{-1}). Most notable is the low glucose level in blood of short-horned sculpin, as previously reported (e.g. MacCormack and Driedzic, 2007). Extracellular glucose sets the upper limit to the diffusion gradient necessary for glucose entry into cells. The selected experimental animals allow an assessment of whether extracellular glucose level influences the mechanisms and rates of glucose trafficking by RBCs.

Methodologies

Rate of glucose uptake (i.e. the passage of glucose across the RBC membrane) was assessed as the initial short-term incorporation of [6-^{14}C]-glucose into the acid-soluble pool. These values represent the maximum potential rate of uptake into cells with minimal levels of intracellular glucose. Uptake of [6-^{14}C]-glucose or glucose analogs is a standard and often-used technique (see Introduction for reference). Here we utilized the natural D-glucose to avoid potential complexities of species differences in the handling of non-metabolizable glucose analogs. The technique measures all radiolabel entering the cell and remaining as glucose or having alternative fates. Under the time courses used in the current experiments, the rate of label lost as $^{14}CO_2$ would be negligible, as in previous work the rate of $^{14}CO_2$ production with isolated RBCs from rainbow trout and sea raven was less than 1% of oxygen consumption (Sephton et al., 1991; Sephton and Driedzic, 1994a).

We encountered high background levels of radioactivity in all cases. This did not represent high rates of glucose uptake over the first few seconds of incubation because there was linearity of uptake for many minutes for RBCs from all species. The nature of the high background is beyond the scope of the present study.

Steady-state glucose metabolism was determined for up to 6 h by measuring the release of ^3H_2O from [2-^{3}H]-glucose. This approach was first developed for mammalian RBCs (Hutton, 1972) and is commonly used in studies of heart metabolism (e.g. Broderick et al., 1992; Rovetto et al., 1975). In isolated rat hearts the method underestimates metabolism by ~20% because of dilution of label at the level of glucose 6-phosphate *via* input of glucose equivalents from the glycogen pool (Neely et al., 1972). Dilution of the specific activity

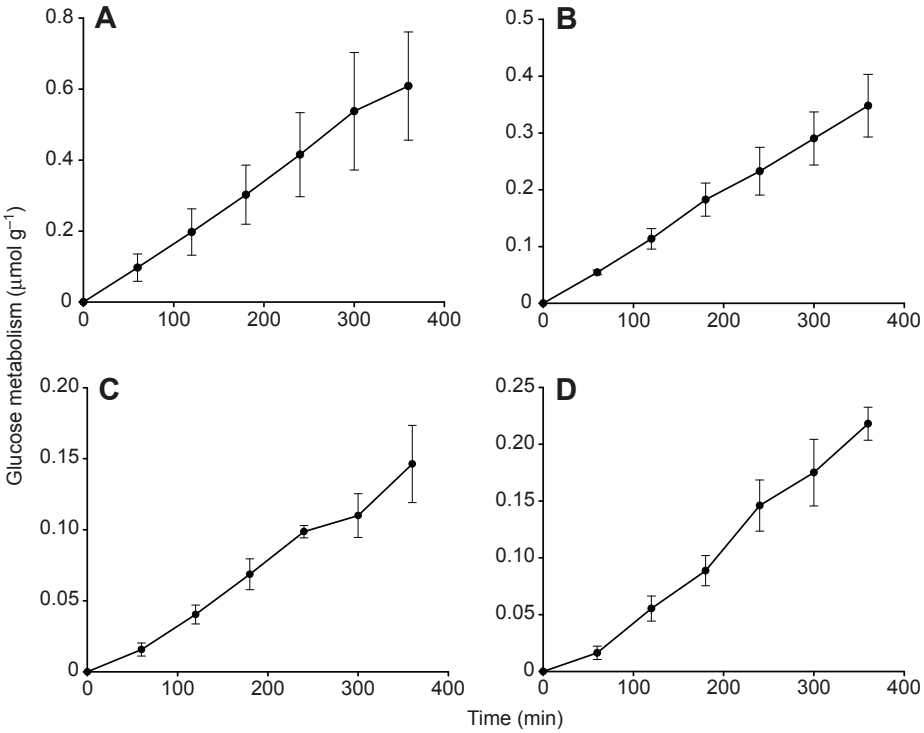

Fig. 5. Time course of ^3H_2O production from [2-^{3}H]-glucose by RBCs from (A) Atlantic cod, (B) Atlantic salmon, (C) cunner and (D) short-horned sculpin. RBCs were incubated with 5 mmol l^{-1} glucose in the extracellular fluid at a temperature of 8°C. *N*=3 for all species.

of the extracellular label presumably occurs in RBCs as well but is unlikely to be substantial as glycogen levels are very low and the incorporation of label from [^{14}C]-glucose into the glycogen pool is trivial compared with that which remains in the acid-soluble pool (Sephton and Driedzic, 1994a). Production of ^3H_2O by RBCs from all four species of this study was linear for at least 6 h. The technique is extremely robust with little background interference.

Evidence for glucose transporters in RBCs of Atlantic cod, cunner and short-horned sculpin

The relationship between extracellular glucose availability and incorporation of [6-^{14}C]-glucose into the acid-soluble pool over a period of 2.5 to 30 min was used as a means of assessing the rates of glucose uptake and the presence of facilitated transport mechanisms. Glucose uptake plateaued for RBCs from Atlantic cod,

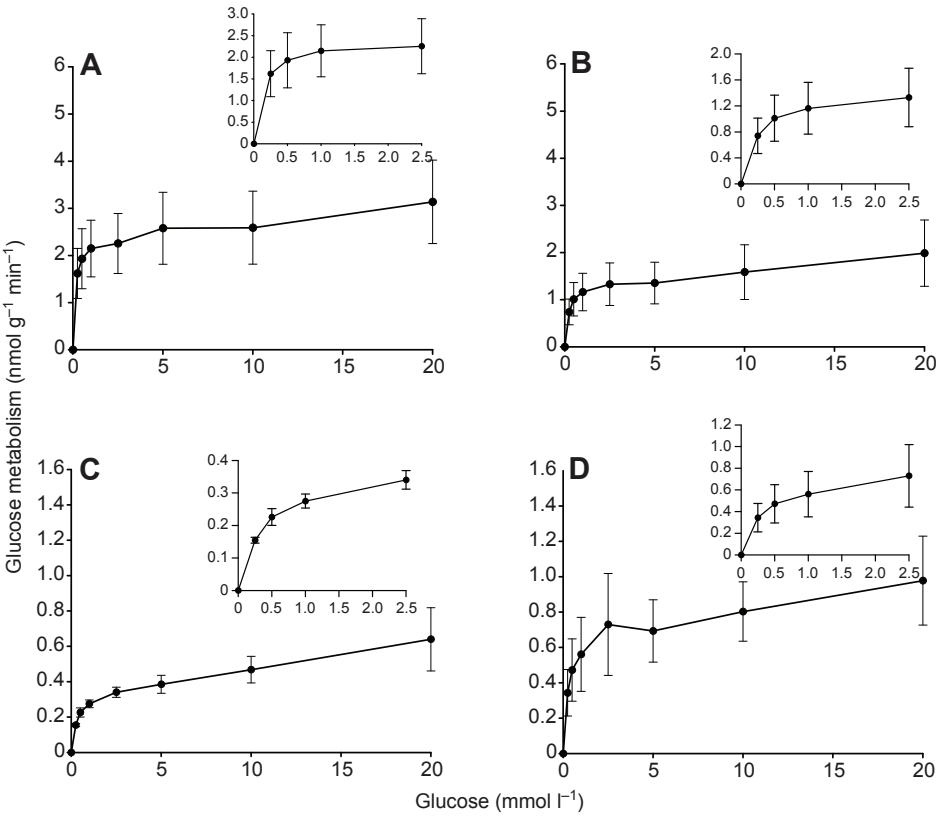

Fig. 6. Relationship between rates of glucose metabolism and extracellular glucose concentration in RBCs from (A) Atlantic cod, (B) Atlantic salmon, (C) cunner and (D) short-horned sculpin. Incubation time was 5 h at a temperature of 8°C. *N*=3 for all species. Insets show the relationship up to a glucose concentration of 2.5 mmol l^{-1}.

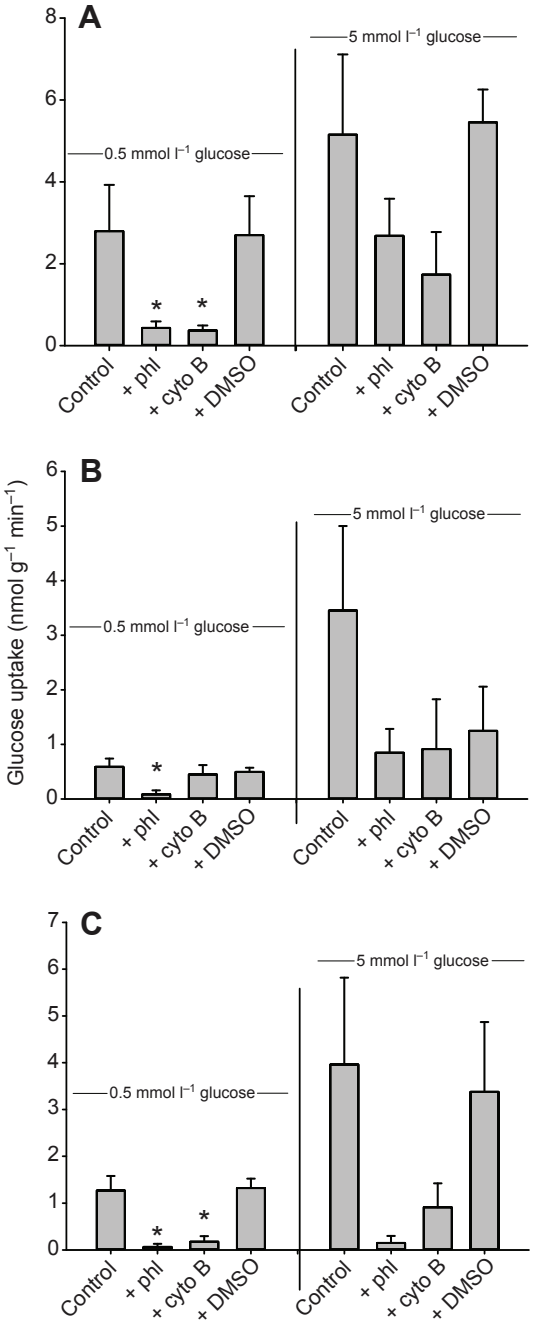

Fig. 7. Impact of phloretin and cytochalasin B on glucose uptake by RBCs from (A) Atlantic cod, (B) cunner and (C) short-horned sculpin. Incubation time was 30 min at a temperature of 8°C. Asterisks indicate a significant difference from control. *N*=3–4 for all conditions.

cunner and short-horned sculpin, suggesting that glucose transporters became saturated. The inhibition of glucose uptake by phloretin and cytochalasin B at low extracellular glucose concentration supports this contention. At higher levels of extracellular glucose, the average level of glucose uptake was always lower in the presence of inhibitors. It is likely that glucose uptake by RBCs from these three species has both a facilitated and a simple diffusive component and at high extracellular glucose the latter would probably take on a greater role. The above interpretation is consistent with arguments for glucose transporters in RBCs of Pacific hagfish (Ingermann et al., 1984; Young et al., 1994), lamprey (Tiihonen and Nikinmaa, 1991a), common eel (Tse and Young, 1990) and American eel (Soengas and Moon, 1995).

Rates of ^3H_2O production were determined at various levels of extracellular glucose as a means of assessing steady-state glucose metabolism. In Atlantic cod, cunner and short-horned sculpin, glycolysis either reached or came close to reaching a maximum at 1 mmol l^{-1} extracellular glucose. This reveals that, within species, the normal physiological level of plasma glucose is not a primary determinant of the rate of glucose metabolism. Instead, the rate of metabolism would be set by the energetic demands. Furthermore, ^3H_2O production was significantly and substantially inhibited by both phloretin and cytochalasin B at both 0.5 mmol l^{-1} (~88% inhibition) and 5 mmol l^{-1} (~74% inhibition) extracellular glucose. The impairment of steady-state glucose metabolism would occur at the level of glucose entry into the cell. This would be consistent with the viewpoint, based on [^{14}C]-glucose uptake, that RBCs from Atlantic cod, cunner and short-horned sculpin all have a facilitated glucose transport system.

Glucose trafficking in salmonid RBCs – a more complex issue

In contrast to the three species discussed above, glucose uptake by Atlantic salmon RBCs was linear up to 20 mmol l^{-1} extracellular glucose. Unfortunately, we were unable to obtain reliable data for glucose uptake in the presence of inhibitors for RBCs from Atlantic salmon. Based on our only available data, using this approach, one could argue that glucose entry occurs by simple diffusion alone in Atlantic salmon RBCs. This was the conclusion reached for rainbow trout RBCs based on the findings that cytochalasin B was without effect on the uptake of OMG at 5 mmol l^{-1} extracellular substrate (Tse and Young, 1990), and for brown trout RBCs, where the uptake of OMG was linear up to 50 mmol l^{-1} extracellular substrate (Pesquero et al., 1992). But as argued below, we consider this position to be oversimplified.

Steady-state glucose metabolism in Atlantic salmon RBCs as measured by ^3H_2O production was unequivocally inhibited by cytochalasin B and phloretin, implying that the presence of glucose facilitated transporters. Numerous tissues including blood of rainbow trout exhibit GLUT1 expression (Teerijoki et al., 2000), and rainbow trout RBCs have the capacity to synthesize protein (Currie and Tufts, 1997; Phillips et al., 2000). As such, salmonid RBCs should have the capacity to generate glucose transporter proteins. The conflicting information for salmonid RBCs is reminiscent of the pioneering studies with birds (reviewed in Carruthers, 1990). Quiescent RBCs show a linear uptake of OMG and are insensitive to cytochalasin B or phloretin treatment, but exposure to agents that increase glucose demand results in a cytochalasin-B-sensitive and saturable component superimposed upon a simple diffusion process. A similar situation may exist in Atlantic salmon RBCs and possibly in the diversity of other species in which it has been argued that glucose entry only occurs by simple diffusion (Kim and Isaacks, 1978; Pesquero et al., 1992; Tiihonen and Nikinmaa, 1991a; Tse and Young, 1990).

Plasma glucose levels in relationship to glucose trafficking

The presence or absence of facilitated glucose transport by fish RBCs is not directly related to plasma glucose levels. This process is apparent in RBCs from species that cover a range of plasma glucose from <1 mmol l^{-1} (short-horned sculpin) to 4 mmol l^{-1} (Atlantic cod). However, there may be a more subtle relationship between glucose transport and extracellular glucose levels. Analysis of glucose uptake

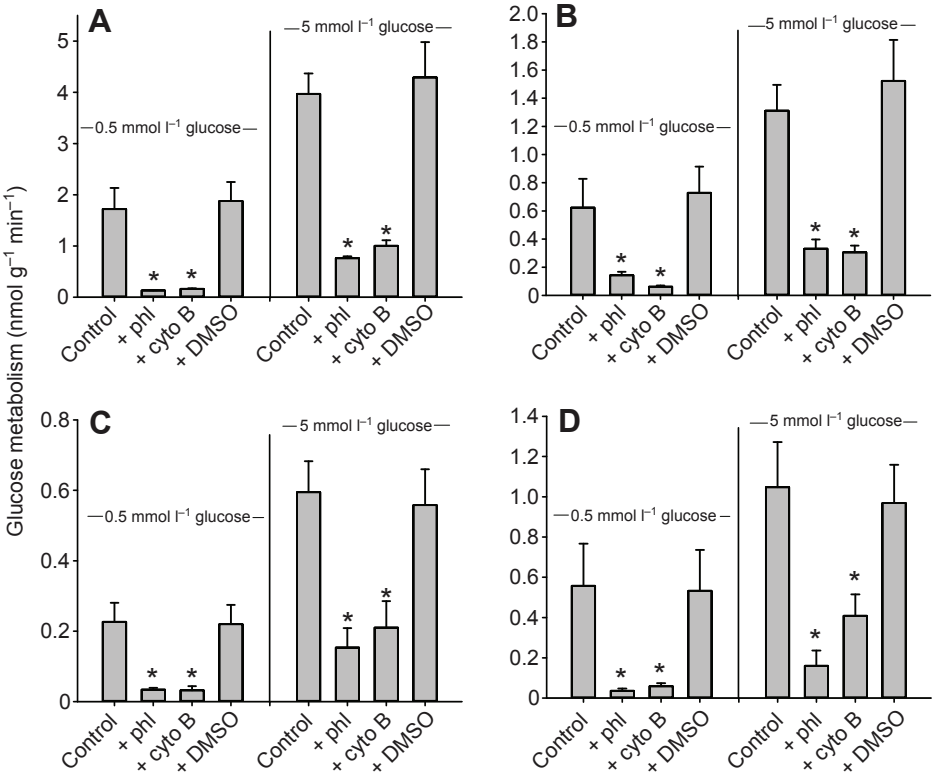

Fig. 8. Impact of phloretin and cytochalasin B on glucose metabolism, as measured by ^3H_2O production from [2-^{3}H]-glucose, by RBCs from (A) Atlantic cod, (B) Atlantic salmon, (C) cunner and (D) short-horned sculpin. Incubation time was 5 h at a temperature of 8°C. Asterisks indicates a significant difference from control. *N*=6 for control and DMSO conditions; *N*=3 for phloretin and cytochalsin B additions.

yielded a K_m of 2.1 mmol l^{-1} for sculpin RBCs with a plasma glucose level of 0.73 mmol l^{-1}. The K_m value is lower than that for Atlantic cod (4.7 mmol l^{-1}) or cunner (7.8 mmol l^{-1}). These data do not suggest a correlation between plasma glucose content and the affinity for glucose but rather that species with exceptionally low glucose levels may have glucose transporters with a particularly high affinity for glucose. The precise nature of the facilitated glucose transporter(s) remains to be resolved. GLUT1 is the only transporter found in mammalian RBCs (Carruthers, 1990) but Atlantic cod RBCs express both a GLUT1 and a GLUT3-like mRNA in a 6:1 ratio (J. R. Hall and W.R.D., in preparation) suggesting that two transporters may be involved. GLUT1 from rainbow trout has a K_m of 8–15 mmol l^{-1} as determined by following uptake of [^{14}C]-glucose into *Xenopus laevis* oocytes (Teerijoki et al., 2001). The similarity with K_m values determined here adds further support to the contention that GLUT1 is a dominant glucose transporter in fish RBCs.

Steady-state rates of glucose metabolism as measured by ^3H_2O production are higher in Atlantic cod and tend to be higher in Atlantic salmon than in cunner or short-horned sculpin. Regardless of plasma levels of glucose, the steady-state rate rate of metabolism under the basal conditions is achieved at close to 1 mmol l^{-1} extracellular glucose, a value that is well below the level of extracellular glucose at which maximal uptake occurs. Presumably the rate of metabolism is set by the energy needs of the cell and not extracellular glucose availability.

Glucose trafficking at the species-specific levels of extracellular glucose is the physiologically relevant situation to consider. Under these conditions, glucose uptake by Atlantic cod is twofold to fivefold higher than the other three species. The major contributing factor to a lower rate by cunner and short-horned sculpin would be the lower level of extracellular glucose, and in the case of Atlantic salmon possibly the number of glucose transporters or a lower affinity for glucose. Glucose metabolism follows a similar species-specific pattern. However, metabolism would be determined by the energy needs of the RBCs and not uptake capacity. At physiological levels of extracellular glucose, the ratio of initial glucose uptake to basal rates of glucose metabolism is between 1.5 and 4 for all species (Fig. 4C). Therefore, for RBCs from all four species there is scope to increase metabolism without any alteration in basal glucose transport capacity, such as an increase in number of membrane-associated transport proteins or modification of the glucose affinity of existing transporters. Activation of hexokinase could decrease the level of intracellular glucose, thus increasing the potential for glucose uptake and increase glycolytic flux. The magnitude of a potential increase in utilization of glucose would be consistent with observations that adrenergic stimulation results in an approximately threefold increase in OMG uptake by brown trout RBCs (Pesquero et al., 1992) and [^{14}C]-glucose incorporation into the acid-soluble pool by rainbow trout RBCs (Sephton and Driedzic, 1994b). This excess capacity could come into play under conditions of increased energy demand due to conditions such as acidosis and associated ion pumping (Ferguson and Boutilier, 1988; Ferguson et al., 1989).

Conclusions

The primary determinants of glucose utilization are: (1) the concentration gradient from the extracellular to the intracellular space (set mostly by extracellular levels), (2) permeation through the cell membrane (enhanced by glucose transporters) and (3) hexokinase activity (regulated by glucose 6-phosphate levels and other signalling processes). Here we assessed the impact of species-specific glucose variability on glucose trafficking in RBCs. Glucose levels in Atlantic cod, Atlantic salmon, cunner and short-horned sculpin are ~4, 4.1, 1.95 and 0.73 mmol l^{-1}, respectively. All species exhibited facilitated glucose transport mechanisms based on the inhibition of glucose metabolism. This and other related evidence, such as the ubiquitous nature of GLUT1 expression, leads us to propose that facilitated glucose transport is common to fish RBCs

despite earlier arguments to the contrary. Under conditions of basal energy demand, an extracellular glucose level of ~1 mmol l^{-1} is sufficient to support glucose metabolism that would be set by metabolic demand as opposed to extracellular glucose level or transport capacity. The ratio of initial glucose uptake/basal glucose metabolism is 2 to 4 for all species at physiological glucose levels that range from 0.75 to 4 mmol l^{-1}. This allows scope for an increase in metabolism, such as would occur during acidosis, without a requirement to alter the glucose transport mechanisms that are already in place.

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