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The regulation and importance of glucose uptake in the isolated Atlantic cod heart: rate-limiting steps and effects of hypoxia

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Summary

This study investigated the regulation of glucose uptake in Atlantic cod (*Gadus morhua*) hearts. Isolated hearts were perfused with or without glucose in the medium, under either normoxic or severely hypoxic conditions. Working at basal levels, hearts did not require extracellular glucose to maintain power under aerobic conditions. However, cardiac performance was significantly reduced without exogenous glucose under oxygen-limiting conditions. The addition of the glucose transporter inhibitor cytochalasin B caused hypoxic hearts to fail early, and hearts perfused with a glucose analogue, 2-deoxyglucose (2-DG), increased glucose uptake 3-fold under hypoxia. The uptake of 2-DG was only partially inhibited when cytochalasin B was added to the medium. Isolated ventricle strips were also incubated in the presence of 2-DG and the extracellular marker mannitol. Glucose uptake (glucose transport plus intracellular phosphorylation) was assessed by measuring the initial rate of 2-deoxyglucose-6-phosphate (2-DG-6-P) accumulation. At 1 mmol l^{-1} 2-DG, the rate of 2-DG uptake remained linear for 60 min, and 2-DG-6-P, but not free 2-DG, accumulation was increased. The fact that intracellular 2-DG did not increase indicates that glucose transport is the rate-limiting step for glucose utilization in non-stimulated cardiac tissue. Replacement of Na^+ by choline in the incubation medium did not affect 2-DG uptake, providing evidence that Na^+-coupled glucose transport is absent in cod cardiac tissue. Similar to cytochalasin B, glucose uptake was also inhibited by phloridzin, suggesting that facilitated, carrier-mediated glucose transport occurs in cod hearts. Under the conditions employed in these experiments, it is clear that (1) activation of glucose transport is required to support hypoxic performance, (2) the rate-limiting step for glucose utilization is glucose transport rather than glucose phosphorylation, (3) 2-DG uptake accurately reflects glucose transport activity and (4) glucose uptake in cod hearts does not involve an Na^+-dependent mechanism.

Key words: glucose uptake, glucose transport, cytochalasin B, 2-deoxyglucose, hypoxia, heart, cardiac performance, cod, *Gadus morhua.*

Introduction

The anatomy of fish is such that a single ventricle pumps blood directly into the gills for oxygenation before it is distributed to the rest of the tissues. The ventricle consists of a spongy endocardium, which in some cases is surrounded by a compact epicardium. Many fish species, including the Atlantic cod (*Gadus morhua*), contain only the spongy layer, which lacks a coronary supply (Farrell and Jones, 1992). Since there are no coronary arteries providing oxygenated blood to the heart, the only metabolic support the heart receives is from the partially oxygenated venous blood returning directly from the systemic circulation to the ventricular lumen (Santer, 1985). The myocytes must acquire oxygen, ions and nutrients from the blood flowing between the trabeculae and therefore are subjected to fluctuating oxygen supply (Satchell, 1991). Oxygen delivery to the heart can be reduced simply by living in conditions of low environmental oxygen or by actively swimming. Previous studies have shown that Atlantic cod hearts are reasonably tolerant to oxygen deprivation. For example, when isolated ventricle strips were subjected to 35 min of anoxia, they continued to perform at ~35% of their initial force at 30 min (MacCormack and Driedzic, 2002). This attribute, along with the lack of coronary arteries, makes this tissue an excellent model system to investigate factors that control anaerobic metabolism, such as glucose uptake under steady-state conditions.

Myocardial glucose metabolism in vertebrates is primarily dependent on the uptake of extracellular glucose. Glucose enters the cell and is phosphorylated by hexokinase to glucose-6-phosphate (G-6-P), which in turn enters the glycolytic pathway common to both glucose breakdown and glycogenolysis. In the rat heart, the maximal *in vitro* activity of hexokinase is about 5-fold greater than the maximal rate of glucose transport (Randle and Tubbs, 1979). Glucose uptake is the rate-limiting step for the mammalian heart under anoxia;

however, under normoxic conditions with insulin present, phosphorylation of glucose to G-6-P becomes rate limiting (Morgan et al., 1961; Cheung et al., 1978) despite high levels of hexokinase. In the mammalian heart, most of the extracellular glucose enters the cell *via* two specific Na^+-independent proteins, GLUT-1 and GLUT-4. GLUT-1 is thought to account for glucose entry under basal conditions, while GLUT-4 is responsive to insulin. Under oxygen-limiting conditions there is translocation of both these transporters from intracellular sites to the sarcolemma, where facilitative diffusion of glucose is realized (Montessuit et al., 1998; Behrooz and Ismail-Beigi, 1999).

The fish heart, similar to the mammalian heart, has the capacity to utilize glucose as a metabolic fuel under aerobic conditions (Lanctin et al., 1980; Driedzic and Hart, 1984; Sidell et al., 1984; West et al., 1993, 1994) and is dependent upon the uptake of extracellular glucose to maintain performance under conditions of oxygen deprivation (Driedzic et al., 1978; Bailey et al., 2000) or elevated work (West et al., 1993). Unlike mammal hearts, how glucose uptake is regulated in fish remains unresolved. Blasco et al. (1996) found that the *in vivo* uptake of 2-deoxyglucose (2-DG) in the heart and brain of the brown trout exceeded the accumulation of 2-deoxyglucose-6-phosphate (2-DG-6-P) by 1.7–2.6 times, although in six other tissues there was a 1:1 ratio. Assuming that 2-DG and glucose are handled in the same fashion, this finding implies that the phosphorylation of glucose, and not glucose transport, is rate limiting in the fish heart even though the *in vitro* activity of hexokinase in fish heart is very high (Driedzic and Gesser, 1994) and greatly exceeds maximal rates of glucose uptake and utilization. This latter characteristic would suggest that transport, and not phosphorylation, is a limiting factor.

The recent cloning and sequencing of glucose transporter (GLUT) proteins in several fish species suggest that facilitative diffusion of glucose into myocytes is the first step for glucose utilization and an important site for metabolic regulation in fish (Planas et al., 2000; Teerijoki et al., 2001). In non-contracting eel ventricle strips, glucose uptake is stimulated under anoxia by about 50%. Treatment of these strips with cytochalasin B (a general inhibitor of glucose transporters) eliminated the anoxia-stimulated component but had no effect on basal transport (Rodnick et al., 1997). These results raise the possibility that another glucose transport mechanism, such as Na^+-dependent glucose uptake, is important in the fish heart. This mechanism has been demonstrated in frog skeletal muscle (Kitasato and Marunaka, 1985) and occurs in other fish tissues such as the kidney (Kanli and Terreros, 1997) and intestine (Reshkin and Ahearn, 1987).

In the current study, we utilized the isolated cod heart and cardiac tissue to: (1) evaluate the impact of GLUT transporter inhibition on performance and glucose uptake under hypoxia; (2) measure glycogen and free glucose levels in hearts subjected to two different oxygen levels and the presence/absence of glucose; (3) test the hypothesis that there is an Na^+-sensitive component to glucose uptake; and (4) examine the relationship between 2-DG uptake and 2-DG-6-P accumulation to assess if uptake or phosphorylation is rate limiting. We conclude that the limiting step in glucose uptake is glucose transport and there is an obligatory requirement for enhanced GLUT activity for hypoxic performance. Na^+-coupled glucose transport is absent and, by default, we propose that a significant component of glucose entry in the teleost heart is based on simple diffusion.

Materials and methods

Animals and reagents

Male and female cultured Atlantic cod (*Gadus morhua* L.) were kept in aerated, flow-through 8100-liter seawater tanks at temperatures between 8°C and 14°C and fed a commercial halibut feed (1–2% of body mass each day; Shur-Gain, Maple Leaf Foods, Inc., Truro, Nova Scotia, Canada). All fish were acclimated for at least 1 month prior to experiments. Cod in the ventricle strip, heart performance and heart glucose uptake studies ranged in mass from 269 g to 759 g (mean ± S.E.M. 529±49 g; N=12), 241 g to 631 g (378±16 g; N=35) and 363 g to 771 g (574±22 g; N=24), respectively. All studies were conducted between August 2001 and October 2002. Unless noted otherwise, all biochemicals were purchased from Sigma Chemical Co. (St Louis, MO, USA).

Preparations

Ventricle strips

Animals were netted individually, killed by a sharp blow to the head, doubly pithed and weighed. The heart was quickly excised and placed in ice-cold medium for marine teleosts (in mmol l^{-1}): 150 NaCl, 5.0 KCl, 1.5 $CaCl_2$, 0.17 $MgSO_4$, 0.17 NaH_2PO_4, 2.33 Na_2HPO_4, 11.0 $NaHCO_3$, 5.0 D-glucose, gassed with 99.5% air:0.5% CO_2, and with pH set to 7.8 at 8°C. After cutting through the pericardium, the entire heart was excised, and the ventricle was dissected free of the atrium and bulbus arteriosus. The ventricle was bisected and four strips (1–1.5 mm wide and 8–10 mm long) were cut by slicing parallel to the long axis of the ventricle with a razor blade. To permit recovery from the effects of tissue slicing, ventricle strips were pre-incubated for 60 min in stoppered 25 ml Erlenmeyer flasks containing 2 ml of basic medium. Medium was supplemented with 35 mmol l^{-1} mannitol and 0.1% bovine serum albumin (BSA) (Cohn Fraction V, essentially fatty acid free). Flasks were gassed continuously with 99.5% air:0.5% CO_2 and incubated at 8°C in a reciprocating incubator (Haake, model SWB 20) at 60 cycles min^{-1} throughout the experiment. Osmolarity was kept constant in all incubations (~340 mosmol l^{-1}) by varying the concentration of mannitol, such that the sum of D-glucose, mannitol, pyruvate and 2-deoxyglucose (2-DG) concentrations was 40 mosmol l^{-1}. Mannitol was also used as a marker of the extracellular space because of its similar diffusion properties to those of glucose; however, unlike glucose, it does not enter the myocyte. To determine whether there is an Na^+-sensitive component of 2-DG uptake in the cod heart, we compared uptake rates in basic

medium with those in Na^+-free medium containing equimolar amounts of choline Cl (150) and choline HCO_3 (11). We also tested whether phloridzin inhibits glucose uptake at a concentration (1 mmol l^{-1}) that significantly inhibits facilitative glucose transport in fish cells (Teerijoki et al., 2001) and mammalian skeletal muscle (Cheng et al., 1978). The water content of ventricle strips (ml g^{-1}) was determined by drying tissue to a constant mass in a vacuum oven set at 70°C.

Perfused hearts

The perfusion medium was identical to the medium used for ventricle strips except for the presence or absence of D-glucose. Temperature of the circulating perfusion medium was maintained at 10°C by connecting the water-jacketed condensers and other chambers to a circulating water bath. D-glucose (5 mmol l^{-1}) was added to the media of several groups including the control groups. Also, one group from each study received 25 μmol l^{-1} cytochalasin B, an inhibitor of facilitative glucose transport proteins (Rodnick et al., 1997). Cytochalasin B was dissolved in DMSO and added to the perfusion medium just prior to the experiment. The final amount of DMSO was 0.025%. Under normoxic conditions, the perfusate was gassed with 99.5% air:0.5% CO_2, whereas severely hypoxic conditions were achieved by gassing with 99.5% N_2:0.5% CO_2. Perfusate oxygen partial pressure (P_{O_2}) was measured by collecting perfusion medium from the input chamber in a glass (Hamilton) syringe and injecting it into a temperature-controlled chamber housing an E101 oxygen electrode (Analytical Sensors Inc., Sugar Land, TX, USA). The oxygen electrode was connected to an OM-200 oxygen meter (Cameron Instrument Company, Port Aransas, TX, USA). P_{O_2} during the stabilization period was 17.8±0.08 kPa. At 5, 15 and 30 min into the perfusion with hypoxic medium, P_{O_2} was 5.87±0.27 kPa, 5.20±0.27 kPa and 4.27±0.27 kPa, respectively.

Performance studies

Cod were killed and their hearts were placed in ice-cold perfusion medium. Hearts were quickly mounted onto the perfusion apparatus as described previously (Driedzic and Bailey, 1994). The apparatus had two water-jacketed condensers. The first condenser was filled with perfusion medium containing 5 mmol l^{-1} glucose and gassed under normoxic conditions. The second condenser was used for experimental treatments and could be gassed with either air or nitrogen mixtures. Gassing of these condensers began approximately 30 min prior to the heart being mounted. The condensers were connected to a water-jacketed filling chamber with side arm, which provided perfusate to the input cannula. The input and output cannula were made of stainless steel tubing and had side arms to allow for pressure measurements. The atrium of the heart was tied onto the input cannula, and the bulbous arteriosus tied onto the output cannula, both by a 2-0 silk ligature. The output cannula was connected to another water-jacketed chamber (output) with side arm set 20 cm above the heart. Overflow from both the input and output chambers was re-circulated back into one of the two condensers.

Once the heart was mounted, perfusion medium from the first condenser (normoxic with glucose) was pumped to the input chamber at a rate that allowed a constant overflow from the side arm and thus a constant input pressure (pre-load, 0.29 kPa). Hearts were electrically stimulated with a Grass Model SD9 square-wave generator set at 5 V and 200 ms duration. Contraction rate was set to 36 beats min^{-1} based on physiological heart rates of 39 beats min^{-1} seen in resting cod subjected to normoxic conditions (Axelsson, 1988). All hearts were forced to work against an imposed after-load of 1.96 kPa. Hearts were kept under these conditions for 15–30 min until flow rates stabilized. After this cardiac parameter had stabilized, perfusion with this medium continued for an additional 10 min before doing any experimental manipulations. This was considered time '0'. The control group (normoxic with glucose) did not have to be switched over to the second condenser. However, the other four experimental conditions (normoxic medium without glucose, hypoxic medium with glucose, hypoxic medium without glucose, and hypoxic medium with added glucose and 25 μmol l^{-1} cytochalasin B) were switched to the second condenser at this time.

All hearts were perfused for an additional 2 h or until hearts failed, as determined by flow less than 1 ml min^{-1}. Once the experiment was terminated, the atrium and bulbous arteriosus were cut away from the ventricle, and the ventricle was cut in half, rinsed with ice-cold glucose-free perfusion medium, blotted and weighed. The two ventricle pieces were frozen in liquid nitrogen and kept in a –80°C freezer for biochemical analysis.

Measurement of 2-DG uptake and extracellular space in ventricle strips

2-DG uptake is defined as transport into the cell and intracellular phosphorylation by hexokinase. Phosphorylated 2-DG is trapped in the myocyte and this makes it possible to measure sugar transport. Uptake measurements were conducted as described previously (Rodnick et al., 1997). After the 10 min rinse step to remove glucose from the extracellular space, ventricle strips were incubated in 1.5 ml of medium containing 2 mmol l^{-1} pyruvate, 1 mmol l^{-1} 2-deoxy-D-[^{3}H(G)]glucose (37 kBq ml^{-1}; specific activity 222 kBq $nmol^{-1}$; New England Nuclear, Guelph, ON, Canada), 37 mmol l^{-1} [U-^{14}C]mannitol (3.7 kBq ml^{-1}; specific activity 2 kBq $nmol^{-1}$; New England Nuclear) and 0.1% BSA for 10–60 min. Radioisotopes were dried under gaseous nitrogen to remove the ethanol and water. Preliminary studies indicated that it takes between 10 and 20 min for full equilibration of the radiolabeled mannitol in the incubation medium with the muscle extracellular space. To terminate experiments, ventricle strips were blotted briefly on filter paper moistened with ice-cold medium, freeze-clamped and stored at the temperature of liquid nitrogen. Strips were weighed and processed by boiling for 10 min in 1 ml of water followed by

centrifugation (1000 ***g*** for 10 min at room temperature). After centrifugation, a portion of the supernatant was counted. The 2-DG and 2-DG-6-P of the muscle extract were separated by ion-exchange chromatography using a small column (0.5 ml bed volume) of DEAE-Sephacel (Jacobs et al., 1990). 2-DG and 2-DG-6-P were eluted with successive washes of distilled water and 0.2 mol l^{-1} HCl, respectively. Duplicate samples of crude muscle extracts, both column fractions and diluted incubation medium were place in scintillation vials containing Ecolume (ICN Biochemicals, Costa Mesa, CA, USA) and counted in a Packard 2500TR liquid scintillation counter with channels preset for dual-label counting. Extracellular space and intracellular concentrations of 2-DG and 2-DG-6-P were determined as described previously for mammalian skeletal muscle (Hansen et al., 1994). The intracellular space was obtained by subtracting the volume of the extracellular fluid from the total tissue water space.

Measurement of 2-DG uptake and extracellular space in perfused hearts

For these experiments, the perfusion system was redesigned in order to decrease the total volume of the perfusion medium and hence reduce the radioisotope requirement. A total volume of 50 ml was achieved by redirecting the overflow from the output chamber directly to the input chamber. This eliminated the need for the two water-jacketed condensers.

Similar to the performance experiments, hearts were mounted and initially subjected to normoxic media containing 5 mmol l^{-1} D-glucose, and flow and output pressure were recorded in order to determine when the preparation was stable. Most preparations stabilized within 30 min. Once stable, the perfusion medium was quickly replaced by a medium containing 5 mmol l^{-1} D-glucose, 2-[^{3}H(G)]-deoxy-D-glucose (18.5 kBq ml^{-1}) and D-[1-^{14}C]-mannitol (5.55 kBq ml^{-1}). [^{14}C]mannitol was added to calculate the extracellular fluid. Once the medium was replaced, it was gassed with either 99.5% air:0.5% CO_2 (normoxic) or 99.5% N_2:0.5% CO_2 (hypoxic). One additional group received a hypoxic medium containing 25 μmol l^{-1} cytochalasin B. P_{O_2} was measured for 20 and 25 min into the stabilization period, 10 min after the switch to radioactive normoxic medium and every 5 min during perfusion with the radioactive hypoxic medium. P_{O_2} during the stabilization period was 17.7±0.08 kPa. 5, 10 and 15 min into the perfusion with hypoxic medium, P_{O_2} was 4.93±0.04 kPa, 2.9±0.27 kPa and 2.13±0.13 kPa, respectively.

After 15 min of perfusing with the radioisotopes, the atrium and bulbous arteriosus were cut away from the ventricle; the ventricle was cut in half, rinsed in ice-cold glucose-free perfusion medium, weighed and frozen in liquid nitrogen. Ventricle samples (~150 mg) were homogenized in nine volumes of 6% perchloric acid, then duplicate samples (200 μl) of homogenate and 10 ml Ecolume were counted for both ^{3}H and ^{14}C.

Extracellular space (ml g^{-1} ventricle) and the intracellular concentration of 2-DG (μmol g^{-1} ventricle) were calculated as previously described (Rodnick et al., 1997). Although it is possible that perfusion times of 15 min would be insufficient for full equilibration of the extracellular marker (mannitol), 15 min was chosen because it was important that the hearts were still functioning at a high level. In the performance experiments, two out of eight hypoxic hearts perfused with medium containing glucose and cytochalasin B failed by 30 min, and half had failed after 40 min. Even if our estimates of glucose uptake in the perfused heart are low, this should not negate the positive effect of hypoxia on glucose uptake and the negative impact of the cytochalasin B treatment.

Instrumentation and data analysis

Input (pre-load) and output (after-load) pressures were read using a Biotronix BL 630 pressure transducer connected to a Biotronix meter. During the performance study, flows were measured using a Biotronix pulsed logic flowmeter (model BL610). However, a Transonic flowmeter (model T206) with a 2N in-line flow probe (Transonic Systems, Inc., Ithaca, NY, USA) was used to measure flows in the glucose uptake studies. Both the pressure transducer and flowmeter were interfaced to a PowerLab computerized system, and data were collected online every 5 min for 30 s using the accompanying software program. Power was calculated using the formula (Driedzic, 1992):

$$\text{Power} = \frac{\text{developed pressure} \times \text{cardiac output}}{\text{heart mass} \times 980/60 \times 10^{-4}}, \qquad (1)$$

where power is measured in mW g^{-1}, pressure is in cmH_2O, cardiac output is in ml min^{-1} and mass is in g. The power of each preparation was normalized as a percentage of stabilization values.

Assays

Glycogen

Approximately 50 mg of ventricle was homogenized in 0.5 ml 30% KOH and boiled for 10 min. Glycogen was precipitated by adding 0.3 ml of 2% aqueous Na_2SO_4 and 2.0 ml absolute ethanol. After the mixture was centrifuged for 10 min at 1500 ***g***, the supernatant was decanted, the pellet washed with 2 ml 66% ethanol and dissolved in 1 ml warm distilled water. An equal volume of 1.2 mol l^{-1} HCl was then added, and the sample heated in a boiling water bath for 2 h. Hydrolysates were frozen in liquid nitrogen and stored at –80°C until analyzed for glucose content.

Glucose assay conditions were based on a procedure modified from Bergmeyer et al. (1974). Briefly, an assay medium was prepared containing 250 mmol l^{-1} imidazole, 5 mmol l^{-1} $MgSO_4$, 10 mmol l^{-1} ATP and 0.8 mmol l^{-1} $NADP^+$. A 100 μl aliquot of the sample was added to a spectrophotometer cuvette and diluted 1:10 with the assay media. 10 μl of glucose-6-phosphate dehydrogenase (G-6-PDH) was added to remove any endogenous G-6-P. Absorbance was read at 340 nm on a DU640 spectrophotometer (Beckman Coulter, Mississauga, ON, Canada) after 10 min. Hexokinase was then added and the absorbance read after 25–30 min.

Free glucose and lactate

Approximately 50 mg of the ventricle was homogenized in nine volumes of 6% perchloric acid. This homogenate was spun at 10 000 ***g*** for 10 min and the supernatant was collected. Free glucose was measured using the modified procedure from Bergmeyer et al. (1974) mentioned above and lactate was assayed using a commercial kit (Sigma # 826-UV).

Statistical analysis

For the performance studies, analyses of variance (ANOVAs) were done at each time interval, and decisions were made with regard to statistical differences between groups using pairwise comparisons, after performing a Bonferroni adjustment. Statistical analysis of the glycogen, glucose and lactate data and glucose uptake measurements was performed using one-way ANOVA followed by Tukey's *post-hoc* test. $P \leq 0.05$ was considered to be statistically significant for all studies.

Results

Performance studies

Mean pressure, cardiac output and absolute power measurements at $t=0$ were 2.6±0.04 kPa, 21.5±1.2 ml min^{-1} kg^{-1} fish and 1.14±0.07 mW g^{-1} (N=36), respectively. There were no significant differences at $t=0$ between groups.

Isolated heart performance under different conditions is shown in Fig. 1. Normoxic hearts perfused with glucose continued to perform for at least 120 min. After 120 min, power output g^{-1} was 88.7±11.1% of the original value. When glucose was excluded from the perfusate, six of seven normoxic hearts also contracted for 120 min and power output was 76.5±9.8% of the original value. This value was not significantly different from the normoxic group receiving glucose. One heart failed after 110 min. Under hypoxic conditions, seven of eight hearts receiving glucose maintained 69.9±8.9% of the initial power for 30 min. After 120 min, however, half the hearts failed and the power output of the remaining four hearts was just 38.3±9.2% of initial values. After 35 min, performance of oxygen-deprived hearts receiving glucose was significantly lower than hearts that were well oxygenated.

Although the exclusion of glucose did not significantly affect the performance of hearts that were well oxygenated, there was a profound effect when glucose was excluded from the hypoxic medium. Hearts performed at a significantly lower level after 20 min when glucose was excluded from the hypoxic medium. At 20 min, one heart had failed and the mean power

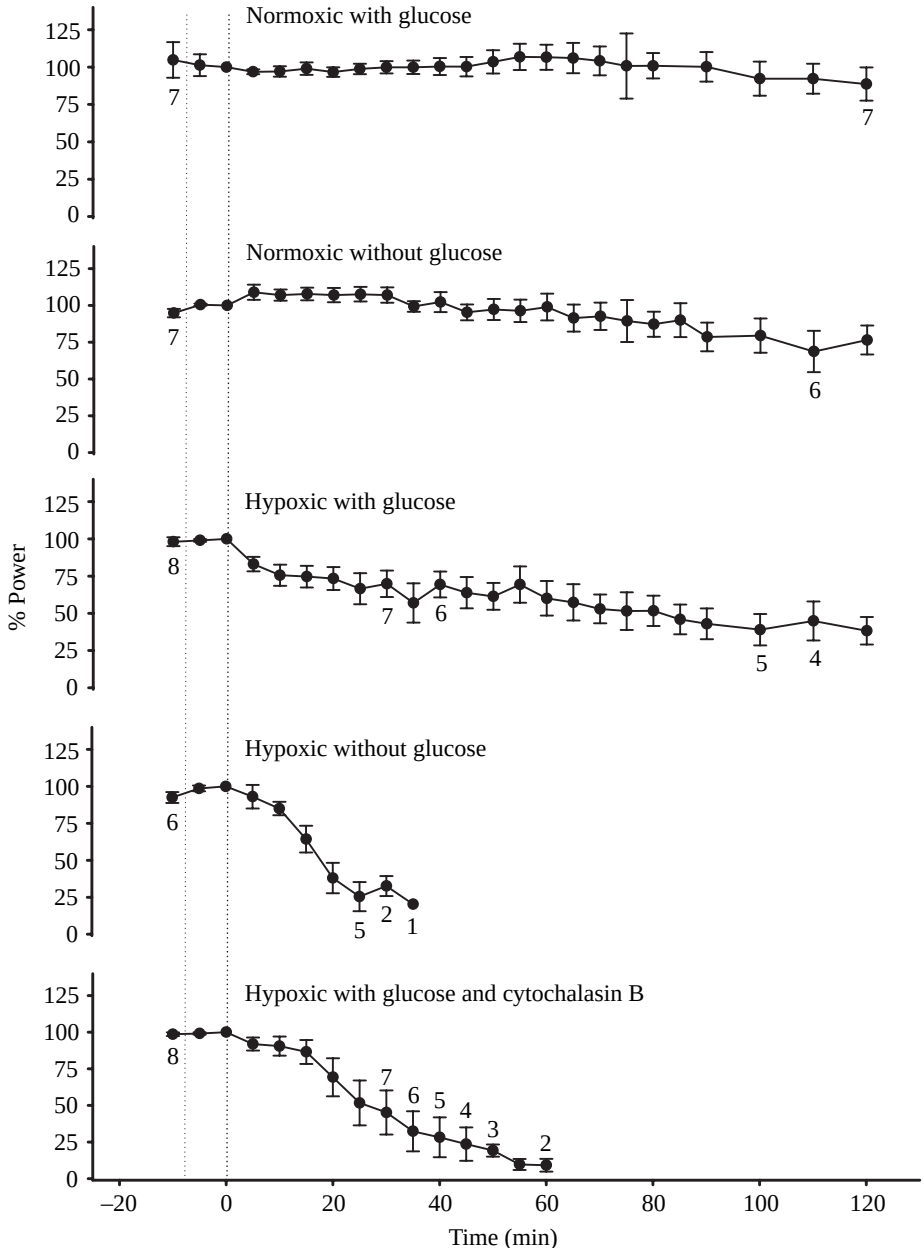

Fig. 1. Power output (%) of isolated cod (*Gadus morhua*) hearts perfused under either normoxic or hypoxic conditions, with or without 5 mmol l^{-1} glucose, or hypoxic conditions with 5 mmol l^{-1} glucose and 25 μmol l^{-1} cytochalasin B in the medium. All values are means ± S.E.M. Numbers under the first data point represent the total number of hearts perfused in that group. Numbers after this initial point correspond to the number of hearts still functioning at that time.

output g^{-1} of the other five hearts was just 38.0±10.3% of initial performance. With glucose in the hypoxic medium, hearts were still performing 73.4±7.6% of the original values after 20 min. Half of these hearts were also still performing after 120 min whereas all hearts perfused with no glucose in the medium failed by 40 min. These observations promote extracellular glucose as an important, if not essential, energy substrate in the working cod heart during hypoxia.

Adding cytochalasin B to the hypoxic medium with glucose caused a significant decrease in heart performance after 40 min when compared to the hypoxic with glucose group (P<0.01). Hearts performed at 69.4±8.7% of basal values after 40 min when perfused with the hypoxic medium (with glucose) but only 28.2±13.6% when cytochalasin B was added. At 40 min, only 50% of these hearts were still functioning, and at 60 min only two of eight hearts continued to develop power at 9.2±4.4% of the initial value. The fact that cytochalasin B binds to the facilitative glucose transporter and inhibits glucose transport activity provides further evidence that extracellular glucose is necessary to maintain heart performance under oxygen-limiting conditions.

Glucose uptake in the perfused heart

Fig. 2 shows the changes in 2-DG uptake in isolated hearts induced by hypoxia or hypoxia and cytochalasin B. Hypoxia stimulated glucose uptake from 1.64±0.12 μmol g^{-1} 15 min^{-1} (normoxic values) to 5.69±0.09 μmol g^{-1} 15 min^{-1} (P<0.01). The addition of 25 μmol l^{-1} cytochalasin B to the hypoxia medium partially inhibited (4.70±0.32 μmol g^{-1} 15 min^{-1}) the stimulation of 2-DG uptake induced by anoxia (P<0.01), but these levels were still higher than the normoxic conditions.

Intracellular concentrations of glycogen, free glucose and lactate

Mean heart glycogen levels were 10.98±1.78 μmol glucose g^{-1} tissue in hearts perfused under normoxic conditions (with glucose). These levels were significantly higher (P≤0.05) than in the hearts perfused under any other condition (Fig. 3A). When hearts were perfused with the hypoxic medium containing glucose, glycogen levels were less than half (4.94±1.57 μmol glucose g^{-1} tissue) the corresponding normoxic values. Similar heart glycogen levels were also seen when glucose was omitted from the normoxic medium (5.23±1.30 μmol glucose g^{-1} tissue). The exclusion of glucose from the hypoxic medium or the addition of cytochalasin B caused heart glycogen levels to drop even lower to 1.27±0.15 μmol glucose g^{-1} tissue and 1.42±0.34 μmol glucose g^{-1} tissue, respectively. These last two groups – hypoxia with no glucose and hypoxia with glucose and cytochalasin B – were not significantly lower than the hypoxic with glucose group or the normoxic without glucose group. Clearly, the absence of exogenous glucose and/or oxygen, or the presence of cytochalasin B, promotes glycogenolysis and the use of endogenous glucose for energy production.

Free glucose in the heart showed a very similar pattern to that of glycogen (Fig. 3B). The highest levels

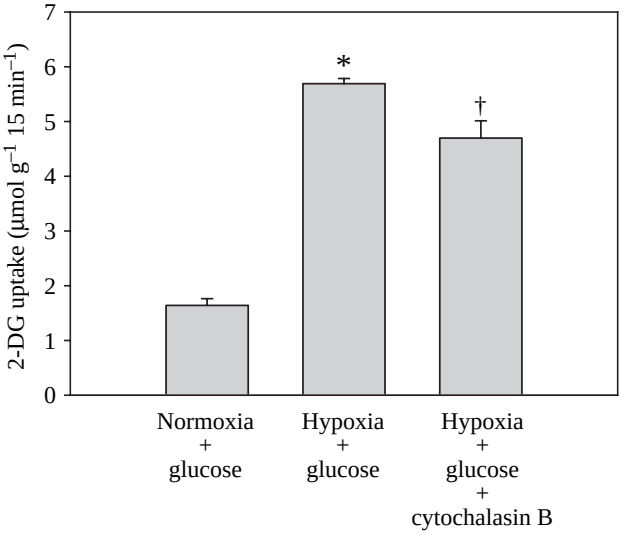

Fig. 2. 2-Deoxyglucose (2-DG) uptake in perfused cod (*Gadus morhua*) hearts. All hearts were perfused for 15 min with 5 mmol l^{-1} glucose in the medium under normoxic or hypoxic conditions. One hypoxic group was also perfused with 25 μmol l^{-1} cytochalasin B in the medium. All values are means ± S.E.M. and N=8 for all groups. *Significantly different from normoxia + glucose group (P<0.001). †Significantly different from both the normoxia + glucose group (P<0.001) and hypoxia + glucose group (P<0.01).

(0.86±0.15 μmol glucose g^{-1} tissue) of free glucose were found in hearts perfused with the normoxic medium with glucose. These levels were significantly higher (P≤0.05) than all other conditions except for the hearts perfused with normoxic medium without glucose (0.48±0.0.12 μmol glucose g^{-1} tissue; P=0.054). Glucose levels were 0.35±0.06 μmol glucose g^{-1} tissue in hearts perfused with a hypoxic with glucose medium. Again, the exclusion of glucose from the hypoxic medium or the addition of cytochalasin B caused the lowest glucose levels found in the heart (0.12±0.03 μmol glucose g^{-1} tissue and 0.20±0.06 μmol glucose g^{-1} tissue, respectively). Lactate levels in the heart did not change under any condition and the overall value was 10.85±0.33 μmol g^{-1} tissue (N=41).

Effects of Na^+-free medium or phloridzin on 2-DG uptake

To define whether there is an Na^+-dependent component of glucose transport in cod cardiac tissue, ventricle strips were incubated in Na^+-free medium. Iso-osmotic replacement of Na^+ with choline had no effect on 2-DG uptake, suggesting that no Na^+-coupled sugar transporters were present (Fig. 4). By contrast, when ventricle strips were exposed to phloridzin (1 mmol l^{-1}), 2-DG uptake was inhibited by 50% (P<0.01). Based on these measurements, facilitative transport of glucose appears to represent a major physiological component of cardiac glucose uptake.

Accumulation of 2-DG and 2-DG-6-P in ventricle strips

Our measures of the extracellular space after 20 min equilibration in ventricle strips from cod

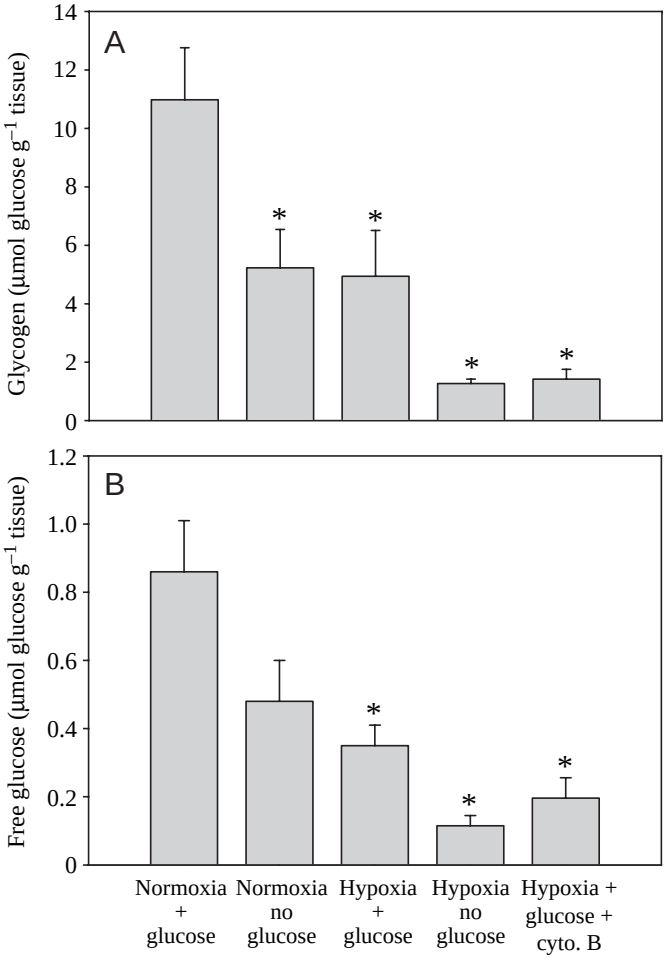

Fig. 3. (A) Glycogen and (B) free glucose levels in the cod (*Gadus morhua*) perfused heart subjected to either normoxic or hypoxic conditions, with or without 5 mmol l^{-1} glucose, or hypoxic conditions with 5 mmol l^{-1} glucose and 25 μmol l^{-1} cytochalasin B in the medium. These levels were taken after 120 min or immediately after the heart failed. All values are means ± S.E.M. and *N*=6–11 fish. *Significantly different from normoxia + glucose group (*P*<0.05). In the normoxic without glucose group, free glucose levels were not significantly different when compared with the normoxic with glucose group (*P*=0.054).

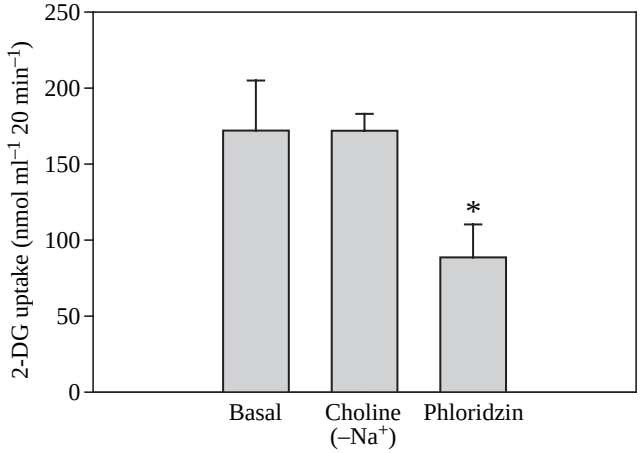

Fig. 4. Effects of Na^+-free medium or phloridzin (1 mmol l^{-1}) on 2-deoxyglucose (2-DG) uptake in cod (*Gadus morhua*) ventricle strips. After dissection, strips were incubated for 60 min in either regular medium (basal condition), Na^+-free medium containing equimolar choline, or regular medium containing 1 mmol l^{-1} phloridzin. All media were supplemented with 5 mmol l^{-1} glucose, 35 mmol l^{-1} mannitol and 0.1% bovine serum albumin (BSA). Ventricle strips were rinsed in glucose-free medium and then assayed for glucose uptake activity in the presence of 1 mmol l^{-1} 2-deoxy-D-[^{3}H(G)]glucose. Values are means ± S.E.M. for six strips per bar. *P<0.01 *versus* basal condition.

(0.219±0.037 ml g^{-1} wet mass; N=12) are comparable to studies of the rat heart using mannitol as the marker (0.236±0.012 g g^{-1} wet mass; Dobson and Cieslar, 1997) and suggest that damage due to cutting the tissue or muscle incubation was minimal. Uptake of 2-DG was linear for 60 min in non-contracting ventricle strips exposed to 1 mmol l^{-1} 2-DG (Fig. 5A), and phosphorylated 2-DG represented the majority of total intracellular 2-DG (Fig. 5B). After a 20 min incubation, free 2-DG was detected in only six out of 12 strips, and the concentration (0.036±0.009 mmol l^{-1} intracellular fluid) was much lower than the extracellular value (1 mmol l^{-1}). After 60 min, free 2-DG was detectable in three of six strips at the concentration of 0.07±0.03 mmol l^{-1}. This concentration represents <10% of the total intracellular 2-DG. These findings provide evidence that, under these conditions, 2-DG uptake rates reflect transport activity, transport is rate-limiting and the accumulation of 2-DG-6-P provides an accurate index of glucose transport activity.

Discussion

The goal of this study was to resolve fundamental questions about the regulation of glucose metabolism in the fish heart. More specifically, we have addressed basic aspects of facilitated glucose transport and the key site of the regulation of glucose utilization at the initial stages of glycolysis.

Heart performance under hypoxia requires facilitated glucose transport

The initial power output (1.14 mW g^{-1}) of perfused cod hearts was slightly lower than reported *in vivo* values (1.77 mW g^{-1}; Axelsson and Nilsson, 1986) and approximately 17% of maximum pumping capacity (6.6 mW g^{-1}) as measured *in situ* (P. C. Mendoça, A. G. Genge, E. J. Deitch and K. Gamperl, unpublished data). Furthermore, the cardiac output of our *in vitro* hearts (21.5±1.2 ml min^{-1} kg^{-1} fish) is well within the range (17–29.1 ml min^{-1} kg^{-1} fish) of those reported for resting cod at 10°C (Jones et al., 1974; Petersson and Nilsson, 1980; Axelsson, 1988). Thus, both these parameters indicate that the perfused hearts were working at similar levels to those of resting cod.

The cod heart does not require extracellular glucose to maintain power under aerobic conditions. Hearts perfused with or without exogenous glucose performed close to 100% of their initial value during the first 60 min. Furthermore, hearts

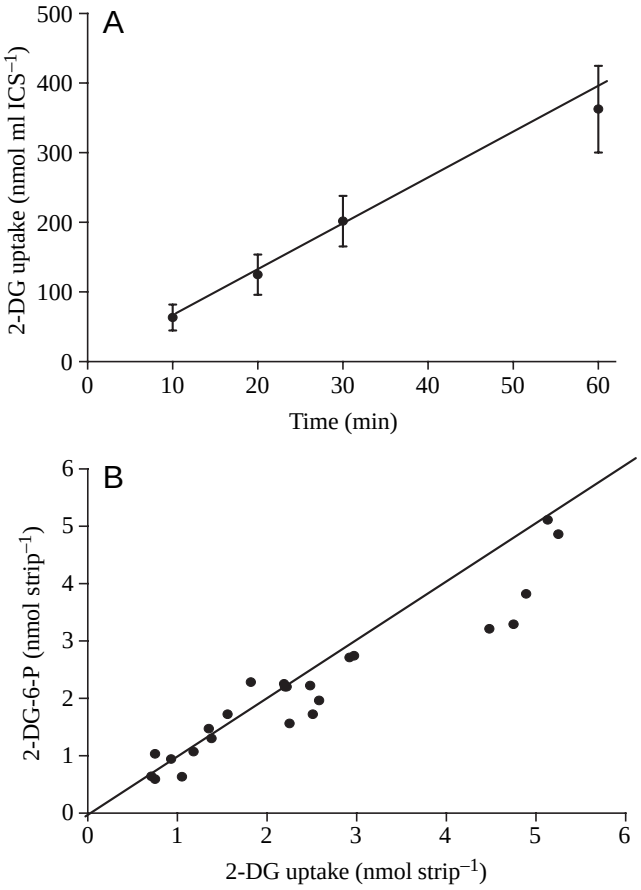

Fig. 5. (A) Time course of 2-deoxyglucose (2-DG) uptake in cod (*Gadus morhua*) ventricle strips incubated with 1 mmol l^{-1} 2-DG. Ventricle strips were incubated at 8°C for 60 min in medium containing 5 mmol l^{-1} glucose, 35 mmol l^{-1} mannitol and 0.1% bovine serum albumin (BSA). Strips were rinsed in glucose-free medium for 10 min and then incubated in medium containing 2 mmol l^{-1} pyruvate, 1 mmol l^{-1} 2-deoxy-D-[^{3}H(G)]glucose, 37 mmol l^{-1} [U-^{14}C]mannitol and 0.1% BSA to assay uptake for the indicated times. ICS indicates intracellular space. Values are means ± S.E.M. for six strips per point. (B) Relationship between 2-DG uptake and 2-deoxyglucose-6-phosphate (2-DG-6-P) accumulation in cod ventricle strips under basal conditions using 1 mmol l^{-1} 2-deoxy-D-[^{3}H(G)]glucose. Strips were frozen after increasing periods of incubation (10–60 min) at 8°C. Intracellular 2-DG and 2-DG-6-P were measured after separation by ion-exchange chromatography into neutral and anionic fractions, respectively. The accumulation of 2-DG-6-P mirrored the rate of 2-DG uptake, whereas free 2-DG represented a minor component. The results are shown for 24 strips from six animals.

perfused with glucose in the medium performed only slightly better during the next 60 min than those hearts perfused without glucose. This confirms previous results indicating that the teleost heart can maintain performance levels with endogenous fuels (Driedzic and Hart, 1984).

Under severe hypoxic conditions, with glucose in the medium, cod hearts were able to maintain a reduced level of performance for periods ranging from 30 to 120 min. This ability to sustain a lower level of performance during severe hypoxia or anoxia has been seen in other fish such as the American eel, bullhead, yellow perch and two species of armored catfish (Tamoatá and Acari-bodó; Bailey et al., 1999). These fish, including the cod, show excellent resistance to the impairment of oxidative phosphorylation, which is indicative of a high level of anaerobic metabolism. With the omission of glucose in the hypoxic medium, cod hearts in this study failed within 40 min. Again, these results are consistent with earlier findings, which showed that an exogenous glucose supply was necessary to maintain force/pressure development in the eel heart under anoxia (Bailey et al., 2000).

At 40 min, the power output of hypoxic hearts perfused with medium containing glucose and cytochalasin B was significantly lower than hearts perfused under hypoxia with glucose in the medium. Since cytochalasin B impairs facilitated glucose transport into cells by binding to glucose transporter proteins (Silverman, 1991), this result provides the first direct evidence that facilitated glucose transport is essential to maintain the contractile performance of the fish heart under oxygen-limiting conditions.

Hypoxia increases glucose uptake but only partly by an increase in facilitated transport

We can only estimate glucose utilization rates in the cod heart because it is unclear if 15 min was sufficient time for the equilibration of the extracellular marker, and a lumped constant that is the ratio of 2-DG uptake to glucose uptake was not determined to correct for differences between tissue uptake of these two compounds. Nevertheless, the rate of glucose uptake under normoxia was similar to that reported for contracting eel ventricle strips (Rodnick et al., 1997) and rainbow trout hearts *in vitro* (West et al., 1993). Hypoxia resulted in a 3-fold increase in glucose uptake compared with normoxic conditions. This increase was much larger than the increase seen in glucose uptake by eel ventricle strips, where the magnitude of the increase was less than 50% of basal rates. It is probable that the difference in the impact of hypoxia/anoxia relates to differences in energy demand by the contracting cod heart *versus* the non-contracting eel ventricle strips. The addition of cytochalasin B to the hypoxic medium in the perfused hearts led to a decrease in the hypoxia-stimulated component of glucose uptake; however, approximately 80% of glucose uptake was insensitive to cytochalasin B. A cytochalasin B-insensitive component to glucose uptake was also observed in the eel heart and was suggested by the authors (Rodnick et al., 1997) to be a result of either membrane damage, a non-transporter-mediated process or reduced affinity of the fish heart glucose transporter for cytochalasin B relative to better-studied mammalian systems. The use of perfused hearts in the current study suggests that tissue damage is not a major problem, leaving the latter two issues still unresolved.

Extracellular glucose provides most of the energy under hypoxia

Severe hypoxia resulted in a significant decrease in glycogen levels in the cod and eel hearts (Bailey et al., 2000). Our data

allow a calculation of the contribution of both extracellular glucose and on-board glycogen to energy production. The rate of 2-DG uptake under hypoxia was ~0.38 μmol g^{-1} min^{-1} (Fig. 2), which on the basis of a net ATP yield of two per glucose molecule equates to 0.75 μmol ATP g^{-1} min^{-1}. This value though will be an underestimate of glucose uptake if 2-DG accumulation underestimates uptake (i.e. a lumped constant less than 1 – see below). The rate of production of glucosyl units from glycogen, under hypoxia with glucose in the medium, can be calculated by the difference in the amount of glycogen between the normoxic with glucose and the hypoxic with glucose groups divided by the average perfusion time. This value was ~0.06 μmol glucosyl unit g^{-1} min^{-1} (Figs 2, 3), which on the basis of a net ATP yield of three per glucose equates to 0.18 μmol ATP g^{-1} min^{-1}. These data reveal for the first time the magnitude of the dependence of the fish heart upon extracellular glucose for the energy production under hypoxia. ATP production from extracellular glucose is minimally about 4-fold greater than that from glycogenolysis. These independent biochemical measures are consistent with the finding of glucose-dependent performance, including the current work with cod and studies with other species (Driedzic et al., 1978; Bailey et al., 2000).

Despite the enhanced utilization of glycogen and myocardial glucose, the amount of lactate (μmol g^{-1} tissue) in the heart did not change under the various conditions. Although not measured, it is presumed that the lactate was released from the heart into the perfusate, the same situation reported for anoxic hearts from the sea raven and ocean pout, where anaerobic metabolism was utilized to support ATP production as indicated by the increased perfusate lactate levels leaving the heart (Turner and Driedzic, 1982).

The absence of an Na^+-sensitive component to glucose uptake

Hexose uptake by teleost cardiac muscle, to our knowledge, has not been examined in the absence of extracellular Na^+. An implication of the present experiments is that Na^+-sensitive glucose transport does not exist in fish cardiac muscle. This contrasts with frog skeletal muscle, where a minor (26%) Na^+-sensitive component of sugar transport has been reported (Kitasato and Marunaka, 1985). The fact that both phloridzin (current study) and cytochalasin B (current study; Rodnick et al., 1997) were effective at inhibiting glucose uptake in fish cardiac muscle promotes facilitative transport of glucose as an important pathway by which sugars are transported across the myocyte sarcolemma. However, the inability to completely block glucose uptake with either of these inhibitors raises new questions about the contribution of other transmembrane pathways and will require further investigation.

Glucose transport and phosphorylation may share control of glucose utilization

Based on our measurements of 2-DG uptake in cod ventricle strips and perfused hearts, we provide new evidence that, in these preparations, membrane transport is rate-limiting for glucose utilization. First, in non-working ventricle strips, the majority of 2-DG was recovered as intracellular 2-DG-6-P, not free 2-DG. Second, the intracellular concentration of 2-DG was much lower than the corresponding extracellular value. Third, concentrations of free glucose in the working perfused heart decreased, not increased, when glucose uptake was increased 3.5-fold by hypoxia. These findings suggest that the rate of glucose phosphorylation is greater than the rate of transport into the cell and that the rate-limiting step in the utilization of extracellular glucose is transmembrane transport. Glucose utilization over a full range of conditions though is possibly more complex than the current point of departure studies reveal.

It was first shown by Morgan et al. (1961) that the major control of glucose uptake in the rat heart shifts between glucose transport and phosphorylation, depending on the experimental conditions. These authors noted an absence of intracellular glucose in isolated rat heart perfused with glucose but no insulin. However, significant amounts of intracellular glucose accumulated in hearts perfused with insulin and glucose above 2 mmol l^{-1} or in hearts under anaerobic conditions receiving at least 4 mmol l^{-l} glucose. Since then, others (Cheung et al., 1978; Manchester et al., 1994; Kashiwaya et al., 1994) have confirmed that glucose transport and phosphorylation alternatively share the control of glucose utilization in the rat heart dependent upon the physiological conditions.

A switching of the prime control site for glucose utilization probably exists in the fish heart as well. Although the current study presents evidence that glucose transport in the cod heart is rate-limiting under both aerobic and hypoxic conditions, experiments with other species suggest that glucose phosphorylation is rate-limiting. For instance, in a study of brown trout *in vivo*, held under normoxic conditions, radiolabeled 2-DG accumulates to a much greater extent than 2-DG-6-P (Blasco et al., 1996). Similarly, increases in heart glucose during hypoxia have been reported for diving African lungfish (*Protopterus aethiopicus*; Dunn et al., 1983), goldfish (*Carassius auratus*; Shoubridge and Hochachka, 1983) and a small Amazon cichlid (*Cichlasoma* sp.; Almeida-Val and Farias, 1996). The contrast between our findings with cod heart and other studies is probably related to species differences and *in vivo versus in vitro* models. Our isolated preparations lack hormonal signals that may activate glucose uptake, in the same way as insulin and epinephrine do in the mammalian heart (Doenst and Taegtmeyer, 1998). In addition, the level of glucose in the medium was maintained at normoxic levels typical for cod. In some species, for example sole (Via et al., 1997), goldfish (Walker and Johansen, 1977) and a species of armored catfish (MacCormack et al., 2003), blood glucose may increase many-fold under hypoxia, which in turn could shift the site of regulation from transport to phosphorylation. The range of blood glucose levels in cod as a function of oxygen availability and other factors has yet to be determined. Additional efforts, both *in vivo* and *in vitro*, will be necessary to better define the conditions that regulate cardiac glucose uptake in fishes that exhibit extreme species variability in blood glucose (from zero to >50 mmol l^{-1}) both within and amongst species (Chavin and Young, 1970; MacCormack et al., 2003).

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