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Fuel use during glycogenesis in rainbow trout (*Oncorhynchus mykiss* Walbaum) white muscle studied *in vitro*

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Summary

The purpose of this study was to examine fuel used during muscle glycogenesis in rainbow trout *Oncorhynchus mykiss* using an *in vitro* muscle slice preparation to test the hypothesis that intracellular lactate is the major glycogenic substrate and the muscle relies upon extracellular substrates for oxidation. Fish were exhaustively exercised to reduce muscle glycogen content, muscle slices were taken from exhausted fish and incubated for 1 h in medium containing various substrates at physiological concentrations. ^{14}C-labeled lactate, glycerol or palmitate was added and ^{14}C incorporation into muscle glycogen and/or CO_2 was measured. Lactate clearance in the absence of net glycogenesis suggests that when suitable oxidizable extracellular substrates were lacking, intracellular lactate was oxidized. Only muscle incubated in lactate, glycerol or palmitate synthesized glycogen, with the greatest synthesis in muscle incubated in lactate plus glycerol. The major fate of these extracellular substrates was oxidative, with lactate oxidized at rates 10 times that of palmitate and 100 times that of glycerol. Neither extracellular lactate nor glycerol contributed significantly to glycogenesis, with lactate carbon contributing less than 0.1% of the total glycogen synthesized, and glycerol less than 0.01%. There was 100 times more extracellular lactate-carbon incorporated into CO_2 than into glycogen. In the presence of extracellular lactate, palmitate or glycerol, intracellular lactate was spared an oxidative fate, allowing it to serve as the primary substrate for *in situ* glycogenesis, with oxidation of extracellular substrates driving ATP synthesis. The primary fate of extracellular lactate is clearly oxidative, while that of intracellular, glycolytically derived lactate is glycogenic, which suggests intracellular compartmentation of lactate metabolism.

Key words: lactate metabolism, white muscle, glycogenesis, rainbow trout, *Oncorhynchus mykiss*.

Introduction

In rainbow trout, high intensity, burst-type exercise is powered primarily by white muscle, fueled by glycogenolysis and anaerobic glycolysis, and fish rapidly exhaust (e.g. Milligan, 1996; Richards et al., 2002a; Richards et al., 2002b). At exhaustion, glycogen, adenosine 5′-triphosphate (ATP) and phosphocreatine (PCr) stores in working muscle are reduced and lactate and H^+ levels are elevated (Richards et al., 2002a). Clearance of anaerobic metabolites and the restoration of energy reserves by muscle are necessary to prepare the fish for subsequent bouts of exercise.

The current model for the restoration of muscle energy reserves in fish after exhaustive exercise suggests that in adult fish glycogen is resynthesized *in situ* with lactate as the main substrate (Milligan and Girard, 1993; Wang et al., 1997; Richards et al., 2002a). Glucose, the main glycogenic substrate in mammalian muscle, is probably not an important glycogenic or oxidative substrate for trout white muscle since muscle has low hexokinase activity (Knox et al., 1980; Storey, 1991) and the expression of glucose transporters in the muscle membrane is extremely low and their physiological role is unclear (Legate et al., 2001; Teerijoki et al., 2001; Capilla et al., 2002). Clearly, if lactate is the main glycogenic substrate and glucose is a relatively unimportant fuel for muscle, other extracellular substrates must be fuelling glycogenesis and muscle metabolism as a whole, during recovery from exercise. The concentration of alanine, a major form of inter-tissue transport of amino acid-derived carbon (Mommsen et al., 1980), increases in both plasma and white muscle following exercise (Milligan, 1997), though its role in fueling muscle recovery from exercise is not known. Overall it is believed that protein does not make a significant contribution to fuelling aerobic metabolism, despite it being plentiful in the white muscle (Lauff and Wood, 1996).

Trout white muscle has considerable intramuscular lipid stores and Richards et al. (Richards et al., 2002a) have suggested a prominent role for lipid oxidation in fueling exercise recovery, thus sparing intracellular lactate carbon for glycogenesis.

Thus, whereas we have some insights into what fuels are probably important for supporting muscle glycogenesis, we have very little understanding of what fuels are preferred and how these fuels are used by muscle (i.e. carbon for glycogen synthesis or for oxidation). Therefore, the purpose of this study was to test the hypothesis that intracellular lactate is the major substrate for trout muscle glycogenesis and the muscle relies upon extracellular substrates for oxidation to provide the ATP necessary to drive glycogen synthesis. We used the *in vitro* isolated white muscle slice preparation, which has been shown to be metabolically viable, capable of glycogenesis and affords the opportunity to control substrate availability (Frolow and Milligan, 2004). Our objective was to determine which substrates support glycogenesis and, using appropriate radiotracers, determine how these extracellular substrates are used by muscle (glycogenic *versus* oxidative fate).

Materials and methods

Experimental animals

Rainbow trout (*Oncorhynchus mykiss* Walbaum; 200–300 g) of both sexes were obtained from Rainbow Springs Trout Farm (Thamesford, Ontario, Canada). Fish were held indoors under flow-through conditions in a 1000 l black circular tank continuously supplied with aerated dechlorinated City of London, ON, Canada water maintained at 15±1°C and a photocycle of 12 h:12 h light:dark. Fish were acclimated to holding conditions at least 3 weeks before use. During holding, fish were fed daily with 2% body weight ration of commercial trout pellets. In order to reduce any dietary effects on metabolism, all fish were isolated in black, acrylic, 4 l boxes, continuously supplied with aerated, water at 15±1°C and fasted for 2 days prior to experimentation (Tang and Boutilier, 1991). All holding and handling of fish was in accordance with the Canada Council on Animal Care Guidelines and approved by the University of Western Ontario Senate Animal Use Subcommittee.

Experimental protocol

Fish were exhaustively exercised by chasing them in a 300 l circular tank for 5 min at which point they were unresponsive to further manual stimulation. Previous studies have shown that this form of exercise leads to exhaustion and a significant reduction in muscle glycogen (e.g. Milligan, 1996). Fish were transferred in a water-filled acrylic, 4 l box to a tank containing 0.3 g MS-222 (tricaine methane sulfonate; Syndel, Vancouver, BC, Canada) buffered to a pH of 7.8 with $NaHCO_3$ in 3 l of dechlorinated tapwater. Fish died within 1–2 min without struggling. Resting fish were killed in the same manner but were not exercised.

Following anesthetization, a 1.5 cm^3 block of muscle tissue of approximately 1.5 g was rapidly excised from epaxial muscle close to the mid-dorsal line of the fish, as described by Frolow and Milligan (Frolow and Milligan, 2004). The block of muscle tissue was then placed in ice-cold modified Cortland's saline containing 140 mmol l^{-1} NaCl, 3.5 mmol l^{-1} KCl, 1.0 mmol l^{-1} $MgSO_4$ $7H_2O$, 3.0 mmol l^{-1} $NaHPO_4$ H_2O, 4.5 mmol l^{-1} $NaHCO_3$, 1.0 mmol l^{-1} $CaCl_2$, 10.0 mmol l^{-1} Hepes and 0.30% defatted bovine serum albumin oxygenated with humidified 99.5% O_2/0.5% CO_2 and adjusted to pH 7.8. Tissue slices of approximately 1.3–1.4 mm in thickness and weighing 243.5±1.8 mg (N=136) were obtained from tissue blocks maintained in iced saline using a Stadie-Riggs microtome and tissue slicer blade (Thomas Scientific, Swedesboro, New Jersey, USA). One slice was immediately blotted dry and frozen by freeze clamping between aluminum blocks cooled with liquid nitrogen to determine the metabolic status of the tissue at the time of sampling (referred to as 'time 0' slice). The remaining slices were incubated in 3.0 ml of modified Cortland's saline (as above) continuously oxygenated with humidified 99.5% O_2/0.5% CO_2 for 1 h at 15°C in a 25 ml Erlenmeyer flask in a shaking water bath. Following incubation, tissue slices were removed, blotted dry, and freeze-clamped. Frozen tissue slices were ground to a fine powder using an insulated mortar and pestle cooled with liquid nitrogen and stored at –80°C until time of analysis.

Experimental series

To quantify the oxidation of extracellular substrates, [^{14}C]CO_2 production was measured using a modified CO_2 trapping system (French et al., 1981) adapted by Walsh et al. (Walsh et al., 1988). A closed CO_2 trapping apparatus consisted of a glass filter paper, wetted with 75 μl of 10 mol l^{-1} benzylthonium hydroxide, placed in a well suspended in a 25 ml Erlenmeyer flask, containing 3.0 ml of aerated incubation medium. Muscle slices were added to incubation flasks, the flasks were sealed and either [U-^{14}C]lactate (specific activity: 50 mCi $mmol^{-1}$; 1 Ci=3.7×10^{10} Bq), [U-^{14}C]glycerol (specific activity: 140 mCi $mmol^{-1}$) or [U-^{14}C]palmitate (specific activity: 400 mCi $mmol^{-1}$) (all from ICN Radiochemicals, Montreal, PQ, Canada) was added to yield the final specific activities in the incubation saline given in Table 1. The substrate concentrations for each experiment are given in Table 1 and are typical levels seen post-exercise in trout plasma (e.g. Richards et al., 2002a). Palmitate was in the form of sodium palmitate, solubilized according the methods described by Berry et al. (Berry et al. 1991). The flasks were incubated in a shaking water bath at 15°C for 1 h. In every experiment, a flask containing everything except tissue was incubated with the experimental flasks to account for spontaneous [^{14}C]O_2 production from the universally labeled ^{14}C substrates and the subsequent [^{14}C]O_2 production rates were corrected accordingly.

After 1 h, 50 μl of 70% $HClO_4$ was added to each flask to halt metabolism and liberate $^{14}CO_2$. The sealed flasks were vigorously shaken at room temperature for 60 min on an orbital shaker at 100 revs min^{-1} to ensure complete collection of [^{14}C]O_2. At the end of this 60 min period, the muscle slices were freeze-clamped and analyzed for muscle glycogen and lactate and the filter papers removed and added to 5.0 ml of Ready Safe (Beckman, Mississauga, Canada) scintillation fluid in a scintillation vial and counted for total ^{14}C radioactivity.

Table 1. *Specific activities used to calculate extracellular lactate, glycerol or palmitate incorporation into tissue glycogen and/or CO_2*

	[^{14}C]lactate		
		d.p.m. g^{-1} tissue	
Incubation conditions	d.p.m. μmol^{-1} saline	Glycogen	CO_2
Lactate (5 mmol l^{-1})	277 686±4820	1024±275	56 450±6430
Lactate (5 mmol l^{-1}) + glycerol (1 mmol l^{-1})	277 686±4820	2020±326	94 830±14 689
Lactate (5 mmol l^{-1}) + palmitate (0.31 mmol l^{-1})	277 686±4820	1102±467	85 502±16 773
Lactate (5 mmol l^{-1}) + palmitate (0.31 mmol l^{-1}) + (1 mmol l^{-1}) glycerol	277 686±4820	730±105	101 048±20 324
Lactate (1 mmol l^{-1})	1 266 649±90 317	2629±396	16 451±2,497
Lactate (1 mmol l^{-1}) + glycerol (1 mmol l^{-1})	1 266 649±90 317	4284±288	29 783±3566
	[^{14}C]glycerol		
Glycerol (1 mmol l^{-1})	284 685±3293	285±51	859±170
Glycerol (1 mmol l^{-1}) + lactate (5 mmol l^{-1})	284 685±3293	307±66	1857±340
	[^{14}C]palmitate		
Palmitate (0.31 mmol l^{-1}) + glycerol (1 mmol l^{-1})	3 518 422±1582	0	107 419±13 336
Palmitate (0.31 mmol l^{-1}) + lactate (5 mmol l^{-1})	3 518 422±1582	0	123 808±8761
Palmitate (0.31 mmol l^{-1}) + lactate (5 mmol l^{-1}) + (1 mmol l^{-1}) glycerol	3 518 422±1582	0	148 195±9547
Palmitate (1 mmol l^{-1})	1 261 840±36 627	0	870±236
Palmitate (1 mmol l^{-1}) + glycerol (1 mmol l^{-1})	1 261 840±36 627	0	4201±648

Values are means ± 1 s.e.m. (*N*=8).

Initial experiments using [^{14}C]$NaHCO_3$ (specific activity: 50 mCi mmol l^{-1}; ICN Radiochemicals) indicated that the [^{14}C]O_2 trapping efficiency of the system is 84±2% (*N*=8) and [^{14}C]O_2 production rates were corrected accordingly.

To determine incorporation of extracellular substrate carbon into glycogen carbon, a 500 μl sample of the tissue glycogen suspension (see below), was added to 5.0 ml of Ready Safe fluor and counted for total ^{14}C radioactivity. For each experiment, each treatment was performed in duplicate on tissue slices obtained from the same fish, and *N* in the figures refers to the number of fish used per experiment.

Analytical techniques and calculations

Frozen tissue slices were individually ground to a fine powder using an insulated mortar and pestle cooled with liquid nitrogen. Muscle glycogen content was assayed by isolating the glycogen (Hassid and Abraham, 1959) and measuring the free glucose after digestion of the glycogen with amyloglucosidase (Bergmeyer, 1965). In our laboratory, we typically recovery 95–100% of the glycogen with this method. Muscle concentrations of lactate, adenosine triphosphate (ATP) and phosphocreatine (PCr) were measured on approximately 50 mg of muscle ground to a fine powder in a liquid N_2-cooled mortar and vigorously resuspended in 1 ml 8% $HClO_4$. Lactate, ATP and PCr were then measured in the supernatant neutralized with 3 mol l^{-1} KOH (Bergmeyer, 1965).

Tissue [^{14}C]glycogen was determined by adding 500 μl of the resuspended glycogen (see above) (Hassid and Abraham, 1959) to 5 ml of Beckman Ready Safe scintillation cocktail. The incorporation of extracellular substrate into the muscle glycogen pool was calculated using the specific activities of the substrate in the saline, and the specific activity of muscle glycogen and CO_2 (Table 1) after Pagnotta and Milligan (Pagnotta and Milligan, 1991) and according to the equation:

$$\frac{\mu mol \text{ substrate incorporated into glycogen or } CO_2}{g \text{ wet tissue}} = \frac{\text{d.p.m. in glycogen or } CO_2 \text{ } g^{-1} \text{ wet tissue}}{\text{substrate specific activity in saline}},$$

where substrate specific activity is in d.p.m. μmol^{-1} in the 3.0 ml incubation saline.

The contribution of extracellular substrate to glycogen synthesis was calculated from the substrate incorporation and the amount of glycogen synthesized over the 1 h period.

All samples were counted on a Packard 1900TR Liquid Scintillation Counter using automatic quench correction. All biochemicals were purchased from Sigma Chemical Co. (Mississauga, ON, Canada), Boehringer-Mannheim Chemical Co. (Laval, PQ, Canada), and all enzymes were purchased from Worthington Biochemical Corp. (Lakewood, NJ, USA).

Statistical analyses

Values presented are means ± 1 s.e.m. (*N*). Statistical analyses were performed using a one-way analysis of variance

Table 2. *The influence of substrate interactions on glycogen synthesis and lactate clearance in muscle slices from exercised fish*

Substrate(s)	Average amount of glycogen synthesized (μmol g^{-1})	Average amount of lactate cleared (μmol g^{-1})
None, saline only	0	13.1±2.8
Lactate (5 mmol l^{-1})	2.4±1.1	10±3.2
Glycerol (1 mmol l^{-1})	2.8±0.8	10.7±2.3
Palmitate (0.31 mmol l^{-1})	2.7±1.1	10.9±2.8
Lactate (5 mmol l^{-1}) + glycerol (1 mmol l^{-1})	5.2±1.8	8.6±1.8
Glycerol (1 mmol l^{-1}) + palmitate (0.31 mmol l^{-1})	4.14±0.8	9.4±2.9
Lactate (5 mmol l^{-1}) + palmitate (0.31 mmol l^{-1})	0	9.8±1.9
Lactate (5 mmol l^{-1}) + glycerol (1 mmol l^{-1}) + palmitate (0.31 mmol l^{-1})	0	9.2±2.1

Muscle slices were incubated for 1 h in the concentration of substrate indicated. Average glycogen synthesized was calculated as the difference between the average at Time 0 (immediately after exercise) level and that at the end of 1 h for only those treatments in which the glycogen content at the end of 1 h was significantly different from that at Time 0 (N=8 for each treatment).

Values are means ± 1 s.e.m.

(ANOVA) followed by Kruskal–Wallis comparison tests or a Student's t-test and significant differences were accepted when $P<0.05$.

Results

At the end of exhaustive exercise white muscle glycogen levels were significantly reduced from resting levels of 9.34±0.75 μmol glycosyl units g wet $tissue^{-1}$ (N=8) to 2.3±0.4 μmol glycosyl units g wet $tissue^{-1}$ (N=8) and lactate levels increased from resting levels of 2.6±1.4 μmol g wet $tissue^{-1}$ (N=8) to 22.5±2.4 μmol g wet $tissue^{-1}$ (N=8). Glycogen synthesis was only seen in muscle slices incubated in lactate, glycerol, palmitate, lactate plus glycerol or glycerol plus palmitate, with the greatest glycogen synthesis seen in the latter two conditions (Table 2). When palmitate and lactate were present together, glycogenesis was inhibited, and there was also no net glycogenesis in slices incubated in lactate, glycerol and palmitate (Table 2). Lactate levels were reduced under all incubation conditions, even in the absence of any net glycogenesis. However, the inclusion of lactate in the incubation mixture appeared to reduce lactate clearance as lactate levels at the end of the 1 h incubation period was consistently higher in those slices incubated in lactate (Table 2).

After 1 h incubation in 5 mmol l^{-1} lactate there was net glycogenesis (Fig. 1A) and significant incorporation of extracellular lactate, as indicated from ^{14}C activity, into both the glycogen and CO_2 pools. The amount of extracellular lactate incorporated into CO_2 was about 100-fold greater than that incorporated into glycogen (Fig. 1B,C). Further, the contribution of extracellular lactate to total glycogen synthesis was trivial, accounting for less than 0.1% of the total glycogen synthesized (compare Fig. 1A and B).

Incubating tissue in glycerol plus lactate increased both net glycogen synthesis and lactate carbon incorporation into glycogen twofold over slices incubated in lactate only (Fig. 1A,B). However, the total contribution of extracellular lactate to glycogenesis was still very small, with only 3.5 nmol g^{-1} wet tissue of extracellular lactate incorporated into the 3500 nmol g^{-1} wet tissue of glycogen synthesized, or about 0.1% of the total. The addition of glycerol also stimulated lactate oxidation as lactate incorporation into the CO_2 pool increased by approximately 1.4-fold (Fig. 1C).

The combination of 0.3 mmol l^{-1} palmitate and 5 mmol l^{-1} lactate did not affect net glycogen synthesis, or lactate incorporation into glycogen or CO_2 compared to either substrate alone (Fig. 1). However, incubation in palmitate plus glycerol plus lactate eliminated the stimulatory effect of glycerol on glycogenesis and lactate oxidation (Fig. 1A,B).

Incubation of muscle slices in glycerol plus lactate consistently stimulated glycogen synthesis compared to that in tissues incubated in glycerol alone (Fig. 2A). However, the incorporation of extracellular glycerol carbon into glycogen synthesized was very small, with only 0.34 nmol g^{-1} entering the glycogen pool, accounting for less than 0.01% of the total glycogen synthesized (Fig. 2B), which is one tenth of the contribution of lactate carbon to glycogen (compare Fig. 1B and Fig. 2B). Extracellular glycerol carbon made a greater contribution to oxidative metabolism than glycogenesis, as there was a tenfold greater incorporation into CO_2 than into glycogen (Fig. 2B,C). However, the oxidation of extracellular glycerol was only 1% that of extracellular lactate oxidation (compare Fig. 1C and Fig. 2C) and was unaffected by the presence of lactate.

Muscle slices incubated with palmitate synthesized glycogen and oxidized palmitate (Fig. 3A,B). Palmitate oxidation was five times that of glycerol, but only at about 10% that of lactate (Fig. 3B *vs* Figs 2C and 1C). Incubation of tissue in palmitate plus glycerol resulted in significantly greater glycogen synthesis, but was without affect on palmitate oxidation (Fig. 3A,B). Incubating tissues in palmitate plus lactate or palmitate plus lactate plus glycerol inhibited glycogenesis, but did not significantly affect palmitate oxidation (Fig. 3A,B).

The substrate concentrations used in the experiments thus

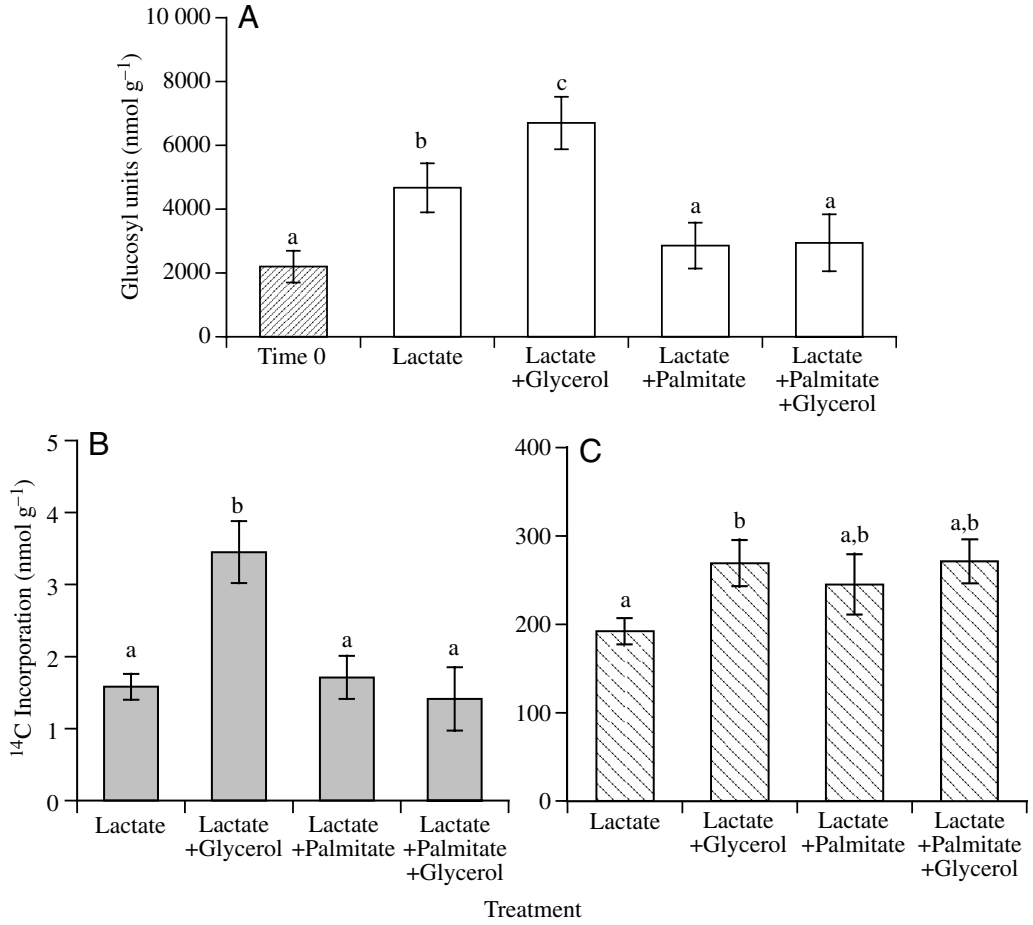

Fig. 1. Glycogenic and oxidative fates of extracellular lactate during glycogenesis in muscle slices from exercised fish. (A) Muscle glycogen levels in tissue sampled immediately after exercise (Time 0) and in tissue incubated for 60 min in the substrates indicated. (B,C) The incorporation of ^{14}C from lactate into the muscle glycogen (B) and CO_2 produced (C). All values are given in nmol g^{-1} wet mass to facilitate comparisons between components. Data are presented as the mean ± 1 s.e.m. for slices from eight different fish (N=8). Columns within the same panel with the same letter are not significantly different from one another.

far were chosen based upon *in vivo* plasma concentrations post-exercise, with up to a 15-fold concentration difference between some substrates (e.g. lactate *vs* palmitate). In order to determine whether the observed differences in substrate utilization reflected actual preferences by recovering muscle or were merely a reflection of concentration differences, a final series of experiments was performed with all substrates at equal concentration, but still within the physiological range.

When all substrates were present at a concentration of 1 mmol l^{-1}, muscle synthesized glycogen (Fig. 4A) and the stimulatory effects of glycerol on glycogenesis in slices incubated in glycerol plus lactate or glycerol plus palmitate were still evident (Fig. 4A). The net amount of glycogen synthesized was also unaffected by the altered substrate concentrations (compare Fig. 4A with Figs 1A, 2A and 3A).

An 80% decrease in extracellular lactate concentration corresponded to an 80% decrease in extracellular lactate carbon incorporation into the glycogen pool, with only 0.3 nmol g^{-1} of lactate carbon incorporated into the glycogen pool as compared with 1.5 nmol g^{-1} at the higher concentration of lactate (compare Figs 4B, 1B). Lowering the concentration of lactate to 1 mmol l^{-1} decreased lactate incorporation into CO_2 by 95% from about 200 nmol g^{-1} to 10 nmol g^{-1} (compare Figs 1C, 4C). However, muscle still exhibited a preference for extracellular lactate oxidation, as ten times more lactate carbon was incorporated into CO_2 than from glycerol and three times more than that from palmitate (Fig. 4C). The stimulatory effect of glycerol on glycogenesis and lactate use was also independent of concentration (Fig. 4A–C). Interestingly, increasing the palmitate concentration by threefold decreased its oxidation by 70% (Fig. 3B *vs* Fig. 4C). As well, in keeping with previous observations, incubation in glycerol plus palmitate resulted in significant glycogen synthesis (Fig. 4A) without altering palmitate oxidation (Fig. 4C).

Discussion

The results of the present study clearly demonstrate that *in vitro*, rainbow trout white muscle is capable of glycogen synthesis, provided it has the appropriate extracellular

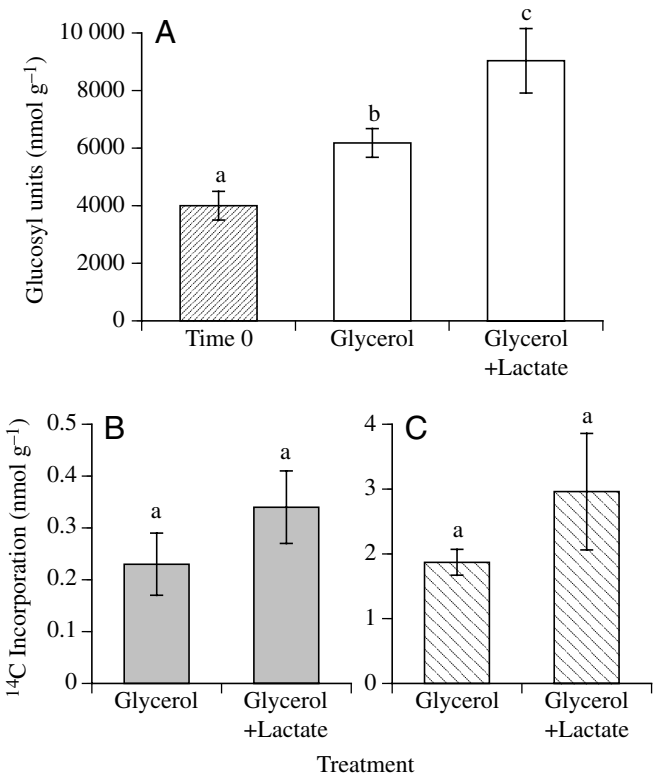

Fig. 2. Glycogenic and oxidative fates of extracellular glycerol during glycogenesis in muscle slices from exercised fish. (A) Muscle glycogen levels in tissue sampled immediately after exercise (Time 0) and in tissue incubated for 60 min in the substrates indicated. (B,C) The incorporation of ^{14}C from glycerol into the muscle glycogen (B) and CO_2 produced (C). All values are given in nmol g^{-1} wet mass to facilitate comparisons between components. Data are presented as the mean ± 1 s.e.m. for slices from eight different fish (*N*=8). Columns within the same panel with the same letter are not significantly different from one another.

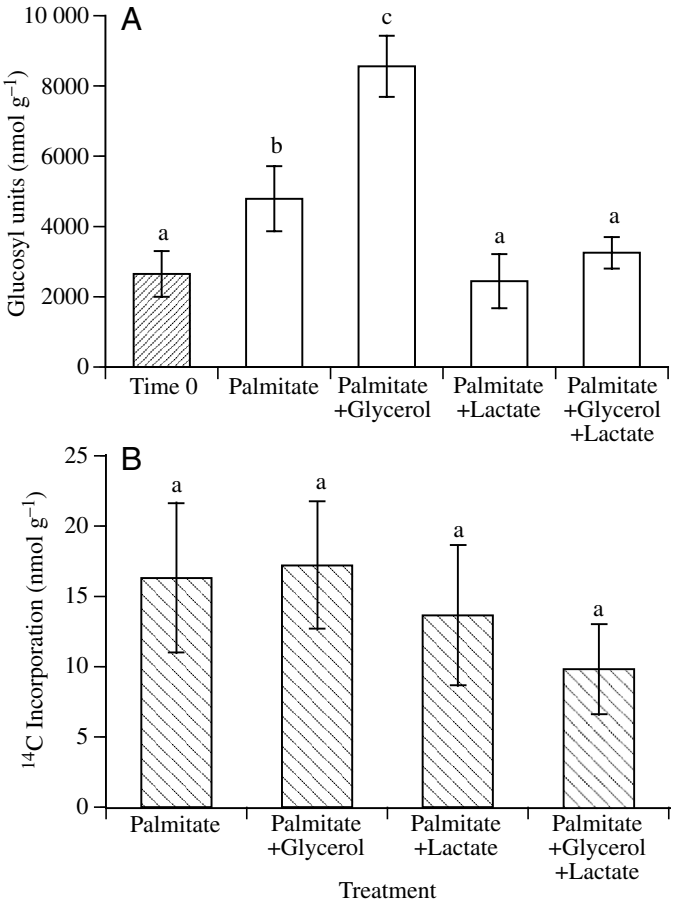

Fig. 3. Glycogen synthesis and palmitate oxidation during glycogenesis in muscle slices from exercised fish. (A) Muscle glycogen levels in tissue sampled immediately after exercise (Time 0) and in tissue incubated for 60 min in the substrates indicated. (B) The incorporation of ^{14}C from palmitate into CO_2 produced. All values are given in nmol g wet mass^{-1} to facilitate comparisons between components. Data are presented as the mean ± 1 s.e.m. for slices from eight different fish (*N*=8). Columns within the same panel with the same letter are not significantly different from one another.

substrates to support oxidative metabolism. Lactate, glycerol and palmitate all stimulated glycogenesis, but the greatest amount of glycogen synthesis was seen in tissues incubated in glycerol plus lactate or glycerol plus palmitate, but when all three were together, there was no net glycogenesis. In all cases, lactate was cleared, with the greatest amount cleared in the absence of net glycogenesis. This is the first study of its kind to examine in any detail how fish muscle uses extracellular substrates to fuel glycogenesis and in the model described below (Fig. 5), we attempt to integrate these new results with what is already known into a model to describe muscle fuel use during glycogen re-synthesis in rainbow trout.

Extracellular substrate use during muscle glycogenesis

In the absence of any extracellular substrates, there was no net glycogen synthesis, despite clearance of a significant amount of muscle lactate, suggesting that under these conditions, the major fate of endogenous lactate is oxidation (Fig. 5 #1). It is unlikely that the disappearance of lactate from the muscle is a result of lactate efflux since the white muscle membrane is relatively impermeant to lactate efflux and as a consequence, the bulk of the lactate generated is retained within the muscle (Sharpe and Milligan, 2003). The presence of extracellular lactate, glycerol or palmitate supported glycogenesis, presumably by serving as oxidative fuels (Fig. 5#2,#3,#4). Their presence probably reduced the reliance upon oxidation of intracellular lactate, sparing some of it for glycogenesis (Fig. 5#5). Although there was net glycogenesis when lactate, palmitate or glycerol was available, the reduction in intracellular lactate was in excess of that accounted for in the amount of glycogen synthesized, suggesting either some intracellular lactate was still being oxidized or the 'missing' lactate (5–6 μmol g^{-1}; Table 2) was trapped in glycogenic intermediates. The observation that when tissues were incubated in glycerol plus lactate or glycerol plus palmitate, all the lactate cleared could be accounted for by the glycogen synthesized could mean that the availability of oxidizable

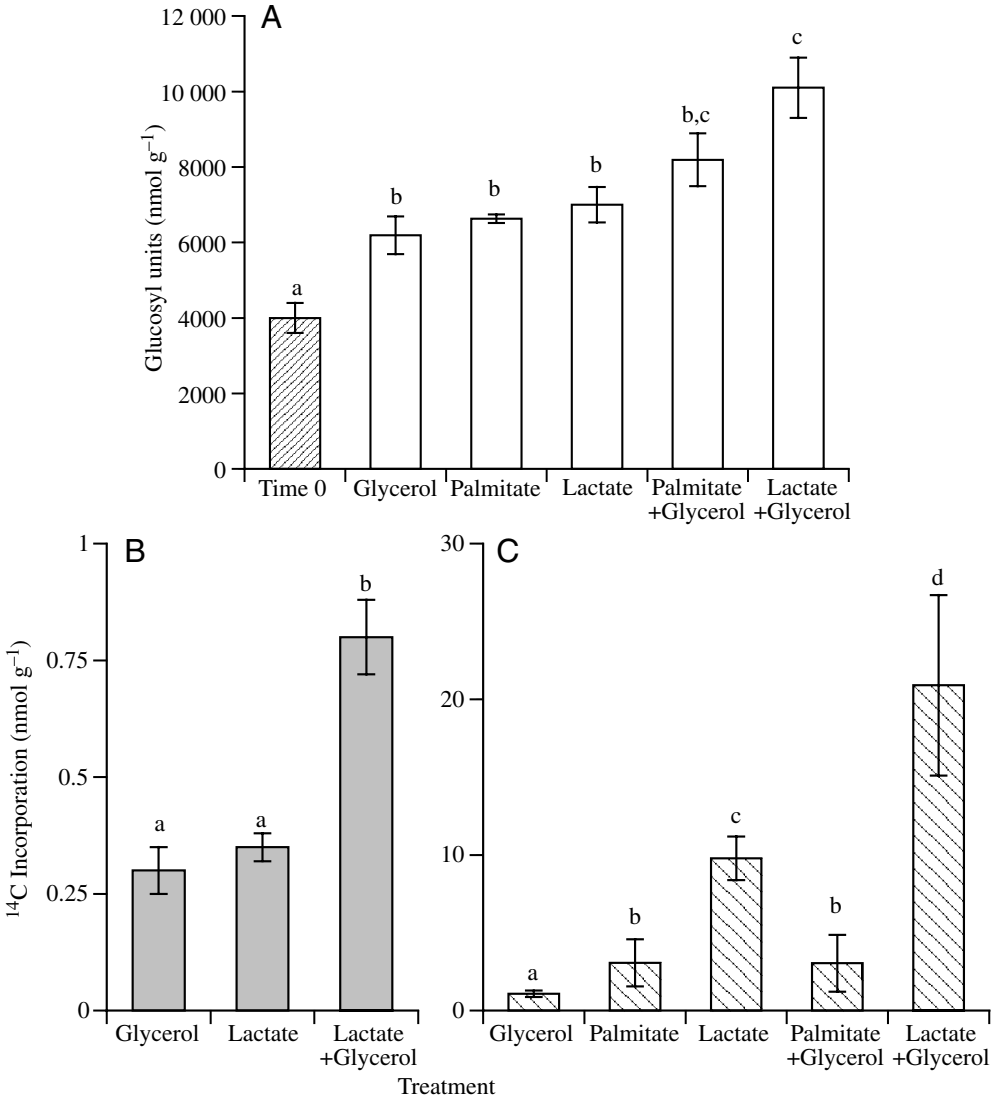

Fig. 4. Muscle glycogen. (A) Glycogen levels in muscle slices obtained from fish immediately after exercise (Time 0), or after 60 min incubation in saline containing various substrates in equal concentration. (B,C) Incorporation of radiolabel from the various substrates into muscle glycogen (B) and CO_2 produced (C). All values are given in nmol g^{-1} wet mass to facilitate comparisons between components. Data are presented as the mean ± 1 s.e.m. for slices from eight different fish (N=8). Columns within the same panel with the same letter are not significantly different from one another.

substrates is limiting to glycogenesis and determines the fate of intracellular lactate.

Lactate was the preferred extracellular substrate for oxidation by muscle during glycogen resynthesis, contributing as much as 16–30% of the ATP required for the observed glycogenesis. This suggest that at least 70% of the ATP needed for glycogenesis came from oxidation of intracellular substrates, probably fatty acids (Richards et al., 2002a). These estimates assume that substrates were completely oxidized (allows calculation of ATP yield) and that glycogen was synthesized from intracellular lactate (allows calculation of ATP requirement). This represents a true muscle preference for extracellular lactate as an oxidizable substrate and not a consequence of differences in substrate concentrations, because when all substrates were present at the same concentration, lactate was still preferred over the other fuels. Lactate is clearly taken up by the muscle, despite the fact that the intracellular lactate concentration was four times that of the extracellular concentration (20 *vs* 5 μmol g^{-1} wet tissue); a gradient that should favor net efflux. Lactate uptake by trout muscle is facilitated by a monocarboxylate (MCT)-like transporter located in the sarcolemmal membrane (Fig. 5#6) (Laberee and Milligan, 1999) and the major fate of this extracellular lactate is clearly oxidative (Figs 1C, 5#7). The amount of extracellular lactate-derived carbon entering the CO_2 pool is about 100 times that entering the glycogen pool (compare Fig. 1B,C) and its oxidation appears to 'spare' lactate of intracellular origin for glycogenesis (Fig. 5#5).

This model suggests that the fate of extracellular lactate is separate from that of intracellular, glycolytically derived

lactate; in other words, lactate metabolism is compartmentalized in trout skeletal muscle. Compartmentation of carbohydrate metabolism has been described for vascular smooth muscle (Lynch and Paul, 1983) in which extracellular glucose was the sole precursor for aerobic glycolysis, whereas glycogenolysis was the precursor for oxidative phosphorylation. Similarly, in insect flight muscle, glycolytic metabolites generated from glucose do not mix with those from glycogen (Srere and Knull, 1998). More recently compartmentation of lactate metabolism has been suggested to explain the simultaneous influx and efflux of lactate in the isolated rat heart (Chatham and Forder, 1996; Chatham et al., 2001). The data from Chatham et al. (Chatham et al., 2001), in which they perfused the rat heart with [3-^{13}C]lactate, indicated that lactate of extracellular origin was preferentially oxidized whereas glycolytically derived lactate (endogenous) was preferentially transported out of the cardiac muscle. These data are consistent with the concept of an intracellular lactate shuttle, first proposed by Stainsby and Brooks (Stainsby and Brooks, 1990), then later refined by Brooks (Brooks, 2000) to include the concept of direct mitochondrial uptake and metabolism of lactate. Although the evidence for the latter component of the intracellular lactate shuttle is conflicting, there is general agreement that some type of compartmentation of lactate metabolism exists in skeletal muscle. Gladden (Gladden, 2004), in a recent review, puts forward the idea of 'microcompartmentation' to explain the different fates of extracellular and intracellular, glycolytically derived lactate. In this model, the physical locations of mitochondria and glycolysis in the cytosol are distinct, such that mitochondria are located primarily at the periphery of the cell, close to the sarcolemmal membrane, the site of extracellular lactate uptake *via* MCTs, and removed from the cytosolic location of glycolysis. Thus, once extracellular lactate is transported into the cell, it is readily converted to pyruvate via cytosolic LDH, which is then transported into the mitochondria and oxidized while the glycolytically derived lactate (intracellular) is localized closer to the glycogenic machinery. Our current understanding of teleost white muscle architecture, in which mitochondria are localized peripherally and the glycogen granules, and presumably the glycolytic and glycogenic enzymes as well, are located between the myofibrils (Sänger and Stoiber, 2001) is consistent with this concept of intracellular compartmentalization of lactate metabolism. Certainly, the fact that the presence of oxidizable extracellular substrates (e.g. lactate, glycerol and palmitate) stimulates glycogenesis and reduces lactate clearance is consistent with the notion that intracellular lactate is 'spared' for glycogenesis and the idea that the enzymatic machinery for glycogenesis is remote from the mitochondria. Clearly, validation of this model awaits further experimentation.

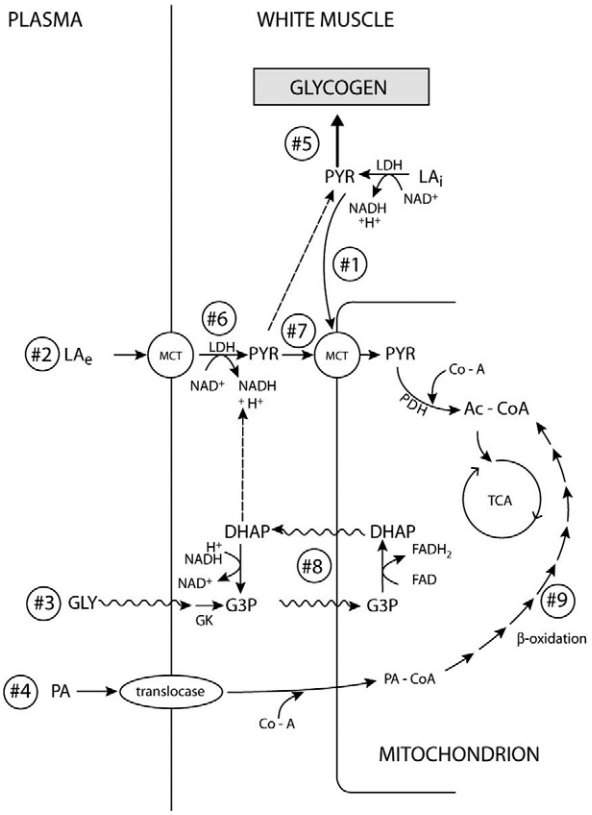

Fig. 5. A proposed model describing extracellular substrate use in support of glycogenesis in trout muscle during recovery from exhaustive exercise. Thicker arrows indicate preferred pathways; wavy arrows indicate diffusion. LA_e, lactate of extracellular origin; LA_i, lactate of intracellular, glycolytic, origin; MCT, monocarboxylate transporter; PYR, pyruvate; LDH, lactate dehydrogenase; PDH, pryruvate dehydrogenase; GLY, glycerol; GK, glycerol kinase; G3P, glyerol 3-phosophate; DHAP, dihydroxyacetone phosphate; PA, palmitate, TCA, tricarboxylic acid cycle; CoA, coenzyme A; Ac-CoA, acetyl CoA; NAD^+, oxidized nicotinamide-adenine dinucleotide; NADH, reduced nicotinamide-adenine dinucleotide; FAD, flavin adenine dinucleotide; $FADH_2$, reduced FAD. See text for details (#, reaction number).

Whereas extracellular glycerol alone can support glycogenesis, its contributions to both oxidative metabolism (its oxidation yields only about 0.4% of the ATP needed for glycogenesis; Fig. 2C) and glycogenesis (glycerol carbon contributed <0.01% to glycogen carbon; Fig. 2B) were minimal. This minimal use of glycerol as an oxidative or glycogenic substrate may reflect low activities of glycerol kinase (GK), which catalyzes glycerol to glycerol 3-phosphate (Fig. 5#8) or an enhanced role of the glycerol 3-phosphate shuttle in fish muscle. The greatest impact of glycerol on muscle glycogenesis was its stimulatory effect on glycogenesis and lactate use when tissues were incubated in glycerol plus lactate. The effect was specific for glycerol on lactate, in that lactate did not influence glycerol use (Fig. 1B,C *vs* Fig. 2B,C). The explanation for this stimulatory effect of glycerol on lactate use and glycogenesis is not clear, but may be related to need for cytosolic NAD^+ to support the first step in lactate metabolism. The glycerol 3-phosphate shuttle, present within mammalian muscle (Fig. 5#8) (Rasmussen et al., 2003), reoxidizes cytosolic NADH to NAD^+, potentially increasing the availability of NAD^+ for lactate metabolism (Hettwer et al.,

2002) and the presence of extracellular glycerol may enhance the activity of the shuttle.

Fatty acid oxidation has been shown to substantially contribute to fueling glycogen re-synthesis during recovery from high-intensity exercise in rainbow trout *in vivo* (Richards et al., 2002a; Richards et al., 2004). Fatty acids of extracellular origin can be used by muscle, as fatty acid uptake has been shown to be carrier-mediated in trout muscle, presumably by a fatty acid translocase that has yet to be characterized. Once in the muscle, these fatty acids undergo β-oxidation (Fig. 5#9) (Richards et al., 2004), supplying the ATP necessary to support glycogenesis, again sparing intracellular lactate for glycogenesis. Accordingly, in the presence of palmitate, there is net glycogenesis and the amount of lactate cleared is reduced. Clearly trout white muscle is able to take up and utilize fatty acids, but the estimated contribution to fueling glycogenesis is somewhat less than suggested *in vivo*. *In vitro*, the contribution of fatty acid oxidation appears to be less important, as palmitate oxidation is only one tenth that of lactate (Fig. 1C *vs* Fig. 3B), and at most, contributed to only 16% of the ATP required to support the associated glycogenesis. Nonetheless, in the presence of palmitate, there was net glycogenesis, an effect that was enhanced when tissues were incubated in glycerol plus palmitate. Glycerol did not stimulate palmitate oxidation, suggesting that the effects are independent of one another. Incubating tissues in lactate plus glycerol plus palmitate had a negative impact on muscle glycogenesis, but was without effect on either lactate or glycerol oxidation. The slight inhibitory effect of lactate on palmitate oxidation is consistent with the inhibitory effects of carbohydrate on fatty acid oxidation (e.g. Richards et al., 2002) and the observation from the present study that lactate is a preferred substrate for muscle oxidative metabolism. However, why the combination of lactate plus palmitate should inhibit glycogenesis is not at all clear. There are reports of palmitate having negative impacts on glycogen synthesis and lactate use in mammalian muscle, but none are consistent with the current observations. For example, in cultured skeletal muscle cells from insulin-resistant diabetic humans, the products of palmitate oxidation have been shown to inhibit fractional glycogen synthase activity presumably because of fatty acid oxidation-induced production of glucosamines (Mott et al., 2000), whereas in the present study, palmitate alone actually stimulated glycogenesis. Furthermore, the addition of extracellular palmitate to the isolated perfused heart from diabetic rats decreases the rate of lactate uptake and oxidation (Chatham et al., 1999), again, not see in this study. The explanation for these negative interactive effects of lactate, palmitate and glycerol on muscle glycogen metabolism is elusive.

The results from this study have been very useful in dissecting out the potential roles and interactions of various extracellular substrates in fueling trout muscle glycogenesis, however, there is a fundamental difference between these *in vitro* and *in vivo* observations that deserves mention. Namely, glycogenesis is more rapid *in vitro* [up to 10 μmol g^{-1} within 1 h post-exercise (Wang et al., 1997; Frolow and Milligan, 2004)] suggesting that mitigating factors exist *in vivo* that act to retard metabolic recovery in muscle. Hormones, in particular cortisol, are absent in theses *in vitro* preparations. *In vivo*, the elevation of plasma cortisol levels, typically seen following exhaustive exercise, appears to be inhibitory to glycogenesis (Milligan, 2003) as no net synthesis of glycogen is seen until cortisol levels begin to decline (Pagnotta et al., 1994). Similar trends were observed *in vitro* in muscle slice preparations (Frolow and Milligan, 2004), though the nature of cortisol's effects are unclear.

In conclusion, the results of the present study provide the basis for generating a comprehensive model describing muscle fuel use during glycogenesis. The hallmark of this model is that muscle exhibits a distinct preference for extracellular lactate, and lactate metabolism is compartmentalized, with the fate of lactate dependent upon whether it is of intracellular (glycolytically derived) or extracellular origin. There are admittedly unknowns in this model, but it provides a framework for further exploration of the metabolic functioning of trout white muscle.

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