Gut glucose metabolism in rainbow trout: implications in glucose homeostasis and glucosensing capacity

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Polakof S, Álvarez R, Soengas JL. Gut glucose metabolism in rainbow trout: implications in glucose homeostasis and glucosensing capacity. Am J Physiol Regul Integr Comp Physiol 299: R19–R32, 2010. First published March 31, 2010; doi:10.1152/ajpregu.00005.2010.—The main objective of the present study was to evaluate the relative contribution of the intestine to glucose homeostasis in rainbow trout. In a first set of in vivo experiments trout were subjected to oral glucose treatments alone or in combination with insulin injections to assess changes in glucose-related enzymes activities, metabolite levels, and mRNA levels. Rainbow trout gut displays an important glucose metabolism that includes the ability to store glucose as glycogen (mostly in the muscle layers) and a large capacity to oxidize glucose. This constitutes a surprising result for a carnivorous fish. In a second set of in vivo experiments, trout received an oral amino acid solution alone or in combination with insulin injection to determine whether other factors besides fasting could regulate gluconeogenesis in intestine. The results confirm the absence of regulation of gluconeogenesis in trout gut, which does not respond to hormones, glucose, lactate, or amino acid changes, either in vivo or in vitro. We also fully characterized gut glucose metabolism in vitro. We observed that a large amount of glucose is oxidized to lactate, supporting the importance of glucose in gut metabolism. Moreover, we corroborated the minor actions of insulin in trout gut, whereas other hormones such as glucagon-like peptide-1 and C-peptide appear to be major hormonal regulators of glucose metabolism in fish gut. Finally, we obtained the first evidence for the existence of a glucosensing mechanism in the midgut of this carnivorous species.

intestine; glucose metabolism; lactate.

GLUCOSE HOMEOSTASIS in carnivorous fish species like rainbow trout (Oncorhynchus mykiss) remains under debate (13, 28). The main tissue controlling glucose homeostasis in fish is the liver, as the main site for glucose production and storage (10). Other organs are also involved in glucose homeostasis, either as glucose producers, such as kidney (52), or as glucose users, such as muscle (21). Fish gastrointestinal tract (GIT) constitutes the first organ that has access to dietary nutrients, and therefore it would be not surprising that its metabolism might be strongly dependent on the type of nutrient absorbed, as well as influenced by diet and nutritional status (35), in a way similar to that observed in mammals (25).

In fish, whereas nutrient transport by GIT has been widely studied and is similar to that observed in other vertebrates (5), much less is known about GIT metabolism. Thus Soengas and Moon (48) in black bullhead or Stokes and Fromm (50) in rainbow trout assessed glucose utilization, whereas Mommsen et al. (27) focused on enzyme zonation in trout, tilapia, and copper rockfish GIT. Finally, Kirchner et al. (18) showed that the gluconeogenic enzymes in trout GIT are unaffected by changes in dietary carbohydrate/protein ratio, with those changes being in part responsible for the persistent postprandial hyperglycemia occurring in this species. On the other hand, whereas in mammals GIT is known to be an insulin-sensitive organ (8) and its metabolism is regulated by numerous hormonal factors, in fish the only hormonal effects reported to date are those of black bullhead enterocytes [insulin, glucagon, and glucagon-like peptide-1 (GLP-1)] (48) and tilapia GIT (cortisol) (27). In mammals, diabetes is accompanied by alterations in the physiological function of the intestine, including increased glucose absorption, glycolytic rates, and glycogen deposition (1, 2). The fact that a carnivorous fish like rainbow trout is considered as a non-insulin-dependent diabates species (28) makes trout intestine an attractive model for glucose metabolism studies.

In mammals, glucose is the most important nutrient with regard to energy supply, and as in other tissues (45), glucosensing capacities also have been reported in GIT cells, including enterocytes, enteroendocrine cells, and enteric neurons (38). Although the mechanism remains to be fully elucidated, some of the actors involved in this function are most likely the same as those present in the glucosensing mechanism of pancreatic β-cells (45), such as glucokinase (GK) or ATP-dependent potassium channels (KATP), whereas others seem to be more GIT specific, such as sodium-dependent glucose transporters (SGLTs) (9, 29). Although glucose is not the main energy substrate for a carnivorous fish, a glucosensing mechanism similar to that described in mammals has been demonstrated in key tissues of rainbow trout that rely on glucose for signal or energy purposes, such as pancreatic and brain tissues (40). However, no data are available to date about the possible existence of a glucosensing system in fish GIT, and information about enteroendocrine and enteric neurons in fish is scarce (3, 32).

Thus the main objective of the present study was to evaluate the relative contribution of the intestine to the glucose homeostasis in rainbow trout. In a first set of in vivo experiments, trout were subjected to oral glucose treatments alone or in combination with insulin injections to assess changes in glucose-related enzymes activities, metabolite levels, and mRNA levels. Based on the lack of response of the gluconeogenic pathway to dietary changes described by Kirchner et al. (18), in a second set of in vivo experiments, trout received an oral amino acid solution alone or in combination with insulin injection. We also carried out different sets of in vitro experiments to fully characterize gut glucose metabolism and to address several questions raised from our in vivo studies.
Accordingly, a first set of experiments was focused on hormone regulation and lactate metabolism, whereas a second set was focused on the potential of amino acids to alter gluconeogenic capacity of intestine. Finally, we also aimed to obtain, for the first time in fish, evidence for the existence of a glucosensing mechanism in the midgut of this carnivorous fish species, and, accordingly, we have identified by molecular and immunohistochemical techniques the presence of potential actors in that putative mechanism.

MATERIALS AND METHODS

Fish

Rainbow trout (O. mykiss Walbaum) were obtained from a local fish farm (Soutorredondo, Spain). Fish were maintained for 1 mo in 100-liter tanks under laboratory conditions and a 12:12-h light-dark photoperiod in dechlorinated tap water at 16°C. Fish mass was 145 ± 7 g. Fish were fed once daily (0900h) to satiety with commercial dry fish pellets (Dibag-Diproteg, Segovia, Spain; approximate food analysis was 48% crude protein, 14% carbohydrates, 25% crude fat, and 11.5% ash; 20.2 MJ/kg feed). The experiments described comply with the Guidelines of the European Union Council (86/609/EU) and the Spanish Government (RD 1201/2005) for the use of animals in research.

In Vivo Experiments

Oral glucose administration. After 1-mo acclimation period, fish were randomly assigned to 100-liter experimental tanks, and each tank was randomly assigned to one of four experimental treatments. Fish were lightly anesthetized with MS-222 (50 mg/l) buffered to pH 7.4 and sodium bicarbonate and then weighed. Eight fish per group then received both oral (O) 10 ml/kg body mass and intraperitoneal (IP) 5 ml/kg treatments consisting of 1) control group: tank water O + saline solution IP; 2) insulin group: tank water O + insulin solution (2 IU/kg; Sigma) IP; 3) glucose group: glucose solution (1 g/ml; Sigma) O + saline solution IP; and 4) insulin + glucose group: glucose solution (1 g/ml; Sigma) O + insulin solution (2 IU/kg; Sigma) IP. Blood and midgut samples were taken 6 h after treatment from fish fasted for 24 h before treatment to ensure basal hormone levels were achieved. Concentrations and times were selected on the basis of studies carried out previously in fish (42, 47).

Oral amino acid administration. Fish were acclimated, distributed, anesthetized, and weighed as described above. Eight fish per group then received both O 10 ml/kg body mass and IP 5 ml/kg treatments consisting of 1) control group: tank water O + saline solution IP; 2) amino acid group: amino acid solution (Aminoplasmal L-12.5; Braun Medical, Barcelonina, Spain) O + saline solution IP; and 3) amino acid + insulin group: amino acid solution O + insulin solution (2 IU/kg; Sigma) IP. Sampling was carried out as described above.

In Vitro Experiments

Every morning of an experiment, fish were dipnetted from the tank, anesthetized with MS-222 (50 mg/l) buffered to pH 7.4 with sodium bicarbonate, euthanized by decapitation, and weighed. The midgut was removed, cleaned of surrounding vessels and fat, opened, and excised in small pieces (60–80 mg). The small pieces were rinsed with modified Cortland medium (141 mM NaCl, 3.5 mM KCl, 4.5 mM NaHCO3, 1.0 mM CaCl2, 1.0 mM MgSO4, 3.0 mM Na2HPO4, 10.0 mM HEPES, 50 U/ml penicillin, and 50 mg/ml streptomycin sulfate, pH 7), sliced in chilled petri dishes, placed in a chilled petri dish containing 100 ml of modified Cortland medium per gram of tissue, and gassed with 0.5% CO2-99.5% O2. The tissue was finely minced and mixed and then placed in 48-well culture plates with 100 ml of modified Hanks’ medium per gram of tissue, gassed with 0.5% CO2-99.5% O2, and incubated (unless indication otherwise) at 16°C for 6 h. The number of independent experiments (n) carried out for enzyme activities was 3 for treatments and 10 for controls, whereas a similar number of experiments were carried out to assess tissue metabolites.

Glucose incubations. Control wells contained modified Cortland medium with 0.5, or 20 mM δ-glucose (Sigma). Treated wells contained medium at the same glucose concentration and one of the selected agents or hormones related to glucose metabolism. These included (final concentration) bovine insulin (10–8 M; Sigma), human glucagon (10–8 M; Sigma), rat GLP-1 (10–9 M, 1–37; Tocris Bioscience), rat C-peptide (10–9 M; Genosys), cytochalasin B (inhibitor of glucose transport through GLUTs) from Helminthosporium dematioides (10 μM; Sigma), and phlorizin (inhibitor of glucose transport through SGLTs, 1 mM; Sigma). In a second set of incubations, two agents related to lactate metabolism were also utilized, such as (final concentration) an inhibitor of lactate dehydrogenase (50 mM sodium oxamate) and an inhibitor of the monocarboxylic acid transporter (1 mM α-cyano-4-hydroxy cinnamate; 4-CIN). All reagents were dissolved in modified Cortland medium, except for cytochalasin B and phlorizin (5% ethanol) and 4-CIN (0.5% ethanol). No effects on the parameters assessed were observed due to the vehicle alone (data not shown). After 6 h of incubation, tissues were quickly removed, frozen in liquid nitrogen, and stored at −80°C until assay.

Amino acid incubations. Control wells contained modified Cortland medium with 5 mM δ-glucose. Treated wells contained medium at the same glucose concentration and Aminoplasmal L-12.5 (Braun Medical) serial dilutions: 1:20 (1X), 1:8 (2.5X), and 1:4 (5X). Osmolality was corrected with NaCl. After 6 h of incubation, tissues were quickly removed, frozen in liquid nitrogen, and stored at −80°C until assay.

Assessment of Metabolite Levels and Enzyme Activities

Plasma and tissue glucose and lactate levels were determined enzymatically using commercial kits (Spinreact, Gerona, Spain) adapted to a microplate format. Samples used to assess metabolite levels were homogenized immediately by ultrasonic disruption in 7.5 vols of ice-cooled 6% perchloric acid and neutralized (using 1 M potassium bicarbonate). The homogenate was centrifuged, and the supernatant was used to assay tissue metabolites. Tissue glycogen levels were assessed using the method of Kepler et al. (17). Glucose obtained after glycogen breakdown (after free glucose levels were subtracted) was determined with a commercial kit.

Samples for enzyme activities were homogenized by ultrasonic disruption with 9 vols of ice-cold-buffer consisting of 50 mM Tris (pH 7.6), 5 mM EDTA, 2 mM 1,4-dithiothreitol, and a protase inhibitor cocktail (Sigma). The homogenate was centrifuged, and the supernant was used immediately for enzyme assays. Enzyme activities were determined using a microplate reader (SPECTRAFluor; Tecan, Grödig, Austria) and microplates. Reaction rates of enzymes were determined as the increase or decrease in absorbance of NAD(P)H at 340 nm. The reactions were started by the addition of supernant (15 μl) at a preestablished protein concentration, omitting the substrate in control wells (final volume 265–295 μl) and allowing the reactions to proceed at 20°C for preestablished times (3–10 min). Enzyme activities are expressed in milligrams of protein. Protein was assayed by using preliminary tests to determine optimal substrate concentrations. Fructose-1,6-bisphosphatase (FPase; EC 3.1.3.11), glucose-6-phosphatase (G6Pase; EC 3.1.3.9), total glycogen synthetase (GCSa) and glycogen synthetase (GCsB; Sigma) as standard. Enzyme analyses were assessed at maximum rates by using preliminary tests to determine optimal substrate concentrations. Fructose-1,6-bisphosphatase (FPase; EC 3.1.3.11), glucose-6-phosphatase (G6Pase; EC 3.1.3.9), total glycogen synthetase (GCSa; EC 2.4.1.11), γ-amylose (EC 3.2.1.3.), lactate dehydrogenase (LDH; EC 1.1.1.27), low-Km hexokinase (HK; EC 2.7.1.1), glucokinase (GK; EC 2.7.1.2), alanine aminotransferase (AAlaAT; EC 2.6.1.1), glutamate dehydrogenase (GDH; EC 1.4.1.2), and pyruvate kinase (PK; EC 2.7.1.40) activities were estimated as described previously (30, 43).
Gene Expression Analysis by Real-Time Quantitative RT-PCR

Total RNA was extracted from rainbow trout midgut using TRIzol reagent (Invitrogen). Total RNA (1 µg) was reverse-transcribed into cDNA using the MMLV reverse transcriptase enzyme (Promega) and random primers (Promega). Gene expression levels were determined by real-time quantitative RT-PCR (qPCR) using the iCycler iQ (Bio-Rad, Hercules, CA). Analyses were performed on 10 µl of the diluted cDNA using the iQ SYBR Green Supermix (Bio-Rad) in a total PCR reaction volume of 25 µl containing 200 nM of each primer. For GK (AF135403), G6Pase (tca00199.d.18_3.1.s.om.8.1–1693), GLUT2 (AF321816), SGLT-1 (AY210436), and β-actin (NM_001124351), qPCR was performed using primers previously described (11, 42). Primers for monocarboxylate transporter-1 (MCT-1), LDH-chain A, and LDH-chain B were designed to overlap an intron, if possible (Primer3 software), by using sequence data from O. mykiss (Sigenae Database) kindly provided by Dr. Stéphane Panserat (INRA, St-Pée-sur-Nivelle, France), as follows: MCT-1 (forward, AGGCTTGGGACTGCGATTCA; reverse, AGCCACGCGACAGGAAAC; Sigenae, CX251492.p.om.7); LDH-chain A (forward, TCCGTGCTAGTGGTGTTGA; reverse, ACACCAGTCCTCCGGACAG; Sigenae, BE859108.p.om.7), and LDH-chain B (forward, TTCCACAGTCAGGCTAATAAGGACA; reverse, TGGCACAGGGGGCTCTTTAC; Sigenae, BE859108.p.om.7). Relative quantification of the target gene transcript was done using β-actin gene expression as reference (34), which was stable expressed in this experiment. Thermal cycling was initiated with incubation at 95°C for 90 s using hot-start iTaq DNA polymerase activation; 35 steps of PCR were performed, each one consisting of heating at 95°C for 20 s for denaturing and at specific annealing and extension temperatures. After the final PCR cycle, melting curves were systematically monitored (55°C temperature gradient at 0.5°C/s from 55 to 94°C) to ensure that only one fragment was amplified. Samples without reverse transcriptase and samples without RNA were run for each reaction as negative controls. Relative quantification of the target gene transcript with the β-actin reference gene transcript was made using the Pfaffl method with the Relative Expression Software tool (REST) (37). This mathematical algorithm computes an expression ratio based on q-PCR efficiency and the crossing point deviation of the unknown sample vs. a control group:

\[ R = \frac{([E_{\text{Target gene}}]^{ACT_{\text{Target gene}}} \text{mean control} - \text{mean unknown sample})}{([E_{\text{β-actin}}]^{ACT_{\text{β-actin}}} \text{mean control} - \text{mean unknown sample})} \]

where E is PCR efficiency determined using a standard curve of cDNA serial dilutions (cDNA dilutions from 1:32 up to 1:512) and ΔCT is the crossing point deviation of an unknown sample vs. a control.

For conventional PCR, amplification of cDNA was achieved with an initial denaturation at 94°C, followed by 35 cycles of denaturation (94°C for 1 min), annealing (60°C for 1 min), and extension (72°C for 1 min), followed by a final extension period of 10 min at 72°C before termination. PCR was carried out in a 20-µl total volume and included 1× PCR buffer, 0.2 mM dNTP, 2 mM MgCl2, 1 unit of Taq polymerase (Invitrogen), 0.2 mM forward and reverse primers, and 1 µl of cDNA. Primers for Kir6.x-like and sulfonylurea receptor (SUR)-like were previously described (41). The PCR products were subjected to electrophoresis in a 1.5% agarose gel. The size of each PCR product was established by comparison with a 50-base step DNA ladder (Promega). Quantification of PCR products was performed by densitometric analysis of ethidium bromide-stained gels using gel documentation system and analysis software.

Immunohistochemical Procedure

Fish were anesthetized with MS-222, and their midgut was extracted. For histochecmy, small pieces fixed by immersion for 24 h at 4°C in Bouin fluid were paraffin-embedded after dehydration treatment. Sections of 6–12 µm thick were then stained using periodic acid-Schiff (PAS) to differentiate glycogen. Control sections were incubated, before staining, in amylase.

For immunohistochemistry, small pieces of intestinal tissues were fixed by immersion in 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS) at pH 7.4 for 24 h at 4°C. Midgut pieces were then cryoprotected in 30% sucrose and embedded in Tissue-Tek OCT (Sakura). Transversal sections (20 µm thick) were made on a cryostat and treated with 3% H2O2 for 30 min and 0.1% BSA for 1 h, to inhibit the endogenous peroxidase activity and nonspecific reactivity, respectively. Next, the sections were then incubated overnight at room temperature in a humid chamber with the following primary antisera raised: GK (rabbit polyclonal; Santa Cruz Biotechnology), SGLT-1 (rabbit polyclonal IgG; Millipore), and GLP-1 (rabbit polyclonal IgG; Santa Cruz Biotechnology). In all, the optimal dilutions were 1:100 in PBS. After several washes in PBS, the sections were then incubated subsequently for 1 h in biotinylated anti-rabbit IgG (Vector) diluted 1:100 in PBS and in ABC kit (Vector) diluted 1:100 in PBS. Finally, the peroxidase reactions were developed in a solution of 3,3'-diaminobenzidine (Sigma; 0.003% in 0.1 M Tris·HCl, pH 7.4) and H2O2 (0.01%). Some developed sections were counterstained with Mayer’s hematoxylin solution. In a control group designed to confirm the specificity of immunostaining, the primary antiseraum was omitted and no immunoreactivity was observed. Rat gut samples were used as positive controls. For attempt colocalization experiments, sections were first incubated with primary antibodies and then with the secondary specific FITC- and TRITC-conjugated antibodies diluted 1:400. Some groups of sections were incubated with antibodies against neuronal markers, acetylated tubulin, human neuronal protein, and calcetin on the basis of previous studies in fish and mammals (32, 33). Slides were observed and photographed under an Olympus microscope (BX51) and digitally photographed (Olympus DP71).

RESULTS

In Vivo Glucose Oral Administrations

Parameter changes as well as metabolite levels and enzyme activities in midgut of rainbow trout subjected to oral administration of glucose combined with insulin are shown in Figs. 1 and 2. As expected, 6 h after oral glucose administration, trout were hyperglycemic (Fig. 1A), exhibiting plasma levels of ~25 mM. On the contrary, fish receiving intraperitoneal insulin administration showed a mild hypoglycemia, with levels about 3 mM. When both glucose and insulin were coadministered, fish remained hyperglycemic, although the degree of hyperglycemia (~17 mM) was lower than in those receiving only glucose treatment. Plasma lactate levels (Fig. 1B) were only altered by insulin treatment, with those fish exhibiting lower lactate levels than the control group. After insulin administration, no changes were noticed in either glucose (Fig. 1C) or glycogen (Fig. 1D) levels in midgut. However, when fish received glucose alone or combined with insulin injection, both parameters increased. It is worth mentioning that the increase was higher for free glucose levels (~10-fold) than for glycogen (~3- to 4-fold). We found that lactate levels in midgut (Fig. 1E) were higher than the control in all the other treatments, but especially with the combination of insulin and glucose, increasing up to threefold more than the saline sham group. Concerning enzymes related to glucose phosphorylation and oxidation, insulin does not seem to regulate their activity, since GK, HK, and PK (Fig. 2, A, B, and J) remained unaltered by treatment with the hormone alone. However, when fish received oral glucose alone or together with insulin injection, we found enhanced glucose phosphorylation and oxidation through glycolysis. Enzymes related to the gluconeogenic potential were differentially regulated: although FBPase activity (Fig. 2C) remained unaltered by any treatment, G6Pase activity (Fig. 2D) was strongly (~4-fold) stimulated by glucose (alone or in combination...
with insulin), whereas the stimulation found with insulin alone was minor. Glycogen-related enzymes were affected by both insulin and glucose: total GSase activity (Fig. 2E) was enhanced by insulin alone and in combination with glucose compared with the control group, whereas α-amylase (Fig. 2F) was also stimulated by insulin alone but, in contrast, was inhibited by the oral glucose treatment (alone or with insulin injection). Finally, LDH activity (Fig. 2G) was enhanced by all treatments, especially by glucose alone.

After oral glucose treatments, mRNA levels of some key proteins involved in glucose and lactate metabolism were also determined (Table 1). Concerning glucose transport, we found that although no altered mRNA levels of GLUT2 were found, increased SGLT-1 mRNA levels were obtained after glucose (alone or with insulin) and insulin treatments. GK mRNA levels were increased after glucose treatment but decreased when insulin was injected. However, the combination of both resulted in a synergistic effect, with mRNA levels increasing up to 40-fold higher than the control group. For G6Pase mRNA levels we found that although glucose stimulates its expression (alone or in combination with insulin), the hormonal treatment alone had an inhibitory effect on mRNA G6Pase levels. On the other hand, MCT-1 mRNA levels were increased by glucose treatment, especially in combination with insulin. Similarly, LDH-A mRNA levels were also enhanced by glucose alone and with insulin injection, whereas LDH-B transcript levels were downregulated by the same treatments.
In Vivo Amino Acid Oral Administrations

Plasma and midgut metabolite levels as well as midgut enzymes activities are shown in Fig. 3. Fish receiving oral administration of amino acid mix (Aminoplasmal) were hypoglycemic (Fig. 3A) compared with those receiving only tank water. Moreover, fish also receiving insulin injection with the amino acid treatment displayed lower glycemia than the other two groups. In contrast to glycemia, plasma lactate levels (Fig. 3B) were differentially regulated by treatments: although the amino acid mix administration caused a decrease in this parameter, the combination with insulin enhanced lactate levels with respect to the control. Midgut glucose (Fig. 3C) and glycogen levels (Fig. 3D) were changed in similar ways when fish were subjected to treatments with decreased levels when the oral amino acid treatment was combined with the insulin injection. In contrast to those results in glucose-related metabolites, G6Pase activity (Fig. 3F) changed in the other direction, being stimulated by insulin plus amino acid treatment compared with control fish. Other parameters, such as midgut lactate levels (Fig. 3E) or FBPase activity (Fig. 3G), were unaffected by the treatments. Finally, activity of enzymes related to amino acid catabolism, such as GDH (Fig. 3H) or AlaAT (Fig. 3I), were affected differentially depending on treatments: GDH activity was only increased with the combination of amino acids and insulin, whereas in contrast, AlaAT was stimulated when only the amino acid mix was administrated orally.

In Vitro Glucose Incubations

The first set of experiments with glucose incubations was done in combination with different hormones involved in glucose metabolism and is shown in Fig. 4. We found that glycogen levels (Fig. 4A) in midgut pieces increased in parallel with the amount of glucose in the medium, from 0 to 20 mM. As a general trend, we observed that insulin and C-peptide
treatment increased glycogen levels, especially at high glucose concentrations, whereas glucagon seems to have had the opposite effect on the level of this metabolite. For GLP-1 incubations we found a dual effect, with increased glycogen levels at 0 and 5 mM glucose but decreased levels at high glucose concentration. Lactate levels (Fig. 4B) in these midgut pieces seemed to follow the glucose concentration in the medium, increasing in concentration in a clear dose-dependent manner with respect to glucose in the wells. This trend was not affected by hormone treatments, although some of them (GLP-1, C-peptide) decreased lactate levels with respect to their control, especially at 5 mM glucose. Both GK (Fig. 4C) and HK activities (Fig. 4D) were affected in similar ways by glucose and hormone treatments. Both increased with the amount of glucose present in the medium, although low-$K_m$ HK activity seemed to be saturated already at 5 mM of glucose, whereas GK also responded to the higher glucose concentration. GK activity was stimulated by glucagon but, in contrast, was inhibited by GLP-1 (undetectable levels with 20 mM of glucose) and C-peptide treatments. Low-$K_m$ HK activity was mainly inhibited by all the hormones tested, especially at high glucose concentrations. However, when the medium was free of glucose, some of the hormones, such as insulin and C-peptide, were able to increase HK activity. PK activity (Fig. 4E) increased with the presence of glucose in the medium, but the increase was similar with 5 or 20 mM glucose. Some of the treatments, such as insulin, glucagon, or C-peptide, increased PK activity when glucose was not in the medium, although most of the treatments resulted in decreased PK activities at 5 and 20 mM glucose. FBPase activity (Fig. 4F) in control group was inhibited by 20 mM glucose. Concerning treatments, C-peptide showed a strong inhibitory effect on FBPase activity at all glucose concentrations; the other hormones assessed enhanced the activity of the enzyme with respect to the control group, especially at 20 mM glucose. LDH activity (Fig. 4G) was unaffected by the glucose treatment in the control group. However, LDH was inhibited by the C-peptide treatment independently of the glucose concentration.

Another set of experiments was carried out to test the transport capacities of the midgut pieces, and thus several metabolic parameters were assessed after incubations with 5 or 20 mM glucose in the presence of two well-known blockers of glucose carriers (cytochalasin B for GLUTs and phlorizin for SGLTs) (Table 2). Concerning metabolite levels, free glucose and lactate levels in the tissue decreased at both glucose concentrations for the two treatments assessed. However, glycogen levels remained unaltered. No low-$K_m$ HK or GK activities were detected at 5 mM glucose when transport was blocked by phlorizin. However, under the other conditions, lower activities than in the control group were found, except for HK with cytochalasin B treatment, which increased. Similarly, PK, LDH, and FBPase activities were generally reduced by the blocking of glucose transport or in some cases remained unchanged.

Data presented in Fig. 5 show lactate studies carried out with midgut pieces incubated with different glucose concentrations at different times. We found that after 5 mM glucose was added to the medium, lactate content in the tissue (Fig. 5A) rose to 2.5–3 mM and remained stabilized at those values during the 6 h of the study. On the other hand, lactate levels increased linearly in the medium (Fig. 5B) during the same period of time ($R^2 = 0.9917$). After this study, pieces were incubated for 6 h with different agents related to lactate metabolism at three glucose concentrations. Thus lactate levels in the tissue increased (Fig. 5C) with glucose concentration in the medium, from 0 to 20 mM glucose. This lactate production (Fig. 5C) was clearly diminished when oxamate was added to the medium, whereas an important increase (up to 2-fold) was noticed when 4-CIN was added. Lactate levels in the medium also increased when glucose levels increased; however, when oxamate or 4-CIN were added to the treatment, the amount of lactate released decreased (Fig. 5D). We carried out several calculations, shown in Fig. 5, E–G, regarding the percentage of lactate produced/exported by tissue. When the medium was free of glucose, the percentage of lactate exported was higher than that produced (Fig. 5E), whereas in the presence of 5 or 20 mM of glucose, this ratio was the opposite (Fig. 5, F and G). When 4-CIN was added to the medium, the amount of lactate in the tissue increased, whereas the exported lactate was reduced, especially if the medium was free of glucose. When oxamate was used as treatment, the amount of lactate released was always higher than that present in the tissue, especially when the medium was free of glucose. Finally, LDH activity ($V_{\text{max}}$; Fig. 5H) was measured to correlate its activity with the lactate production. LDH activity was not affected by the presence of glucose in the medium. However, its activity correlated with the glucose concentration in the medium when oxamate was in the medium, although activities were lower than in the control at 0 mM glucose and higher at 5 or 20 mM glucose. LDH activity was only lower than the control when glucose was not added to the medium.

In Vitro Amino Acid Incubations

Results obtained from midgut pieces incubated with 5 mM glucose and increasing amino acid concentration in the medium are shown in Fig. 6. Lactate levels (Fig. 6A) in the tissue were only affected by amino acid at 2.5× concentration, showing higher levels than the control (0×). Glycogen levels (Fig. 6B) were more affected by treatment, and lower levels

### Table 1. Effects of IP administration of saline or insulin in fish orally receiving tank water or glucose solution on mRNA expression levels of encoding midgut genes

<table>
<thead>
<tr>
<th>Glucose Oral</th>
<th>Insulin IP</th>
<th>Insulin IP–Glucose Oral</th>
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<tr>
<td><strong>Glucose metabolism</strong></td>
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<tr>
<td>GLUT2</td>
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<td>G6Pase</td>
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<td><strong>Lactate metabolism</strong></td>
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<td>+1.02*</td>
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<tr>
<td>LDH-B</td>
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Results are means ($n = 6$) expressed as fold variation of the intraperitoneal (IP) saline injection-treated group and were analyzed by 1-way ANOVA followed by the Student-Newman-Keuls comparison test. *$P < 0.05$, significantly different from control group (saline IP-tank water oral). Different letters indicate significant difference among treatment groups. GLUT2, glucose facilitative transporter type 2; SGLT-1, sodium-dependent glucose cotransporter type 1; GK, glucokinase; G6Pase, glucose-6-phosphatase; MCT-1, monocarboxylate transporter type 1; LDH-A, lactate dehydrogenase, chain A; LDH-B, lactate dehydrogenase, chain B.
than in the control group were found when the amino acid concentration was 1 × or 2.5 ×. Concerning enzymes activities, whereas FBPase activity (Fig. 6C) was inhibited as amino acid levels increased in the medium, GDH (Fig. 6D) and AlaAT activities (Fig. 6E) increased with amino acid concentration (the second in a dose-dependent manner).

At the top of Fig. 7, gene expression of main proteins involved in the glucosensing mechanism in mammals and fish are shown. The histological study shows the muscle and epithelial layers of midgut strongly stained following the PAS techniques (Fig. 7A). However, the PAS staining in the muscle layer disappeared after treatment with amylase, indicating the presence of glycogen (Fig. 7B). As shown in Fig. 7C, the high glycogen accumulation was observed in the cytoplasm of muscle cells. Immunohistochemical tests with antiserum against GK and GLP-1 revealed scarce spindle-shaped positive endocrine cells (Fig. 7, D and G). Positive cells were observed (distributed) in the upper half of the intestinal folds (mucosal folds). In addition, immunoreactivity for GK is present in nerve cells and fibers in the submucosa and in the connective axis of the mucosal folds, i.e., lamina propria (Fig. 7, E and F). The immunoreactivity for GLP-1 and SGLT-1 was always local-
ized in the absorptive cells or enterocytes (Fig. 7, H and I) and in subtle elements (unknown cells) present in the lamina propria.

DISCUSSION

Although the contribution of liver, kidney, or muscle to glucose homeostasis in carnivorous fish is well known, the relative contribution of the intestine remains to be elucidated. In mammals, it is well known that the gut can contribute to the control of glucose homeostasis by its high glycolytic capacity and a recently described gluconeogenic function (22, 23).

Glucose Metabolism in Trout Gut In Vivo

Glucose homeostasis in fish has been often studied using oral glucose challenges (28), but in most of those studies the role of the intestine in glucose homeostasis was ignored. In this study we present data demonstrating that the trout gut is a metabolically active tissue, able to transport, use, and store glucose. The metabolic changes we found suggest that trout gut is able to regulate its own glucose homeostasis.

After subjecting trout to glucose oral treatment (alone or combined with intraperitoneally injected insulin), we found a global metabolic response in gut characterized by increased glycogen and glucose levels, increased glycolytic potential, and unaltered gluconeogenic potential. Both glucose and glycogen levels in midgut paralleled the changes found in glycemia, indicating that the tissue was taking up large amounts of glucose and that some of that glucose is stored in the same tissue, most likely in muscle cells. This is the first time that this function has been attributed to a lower vertebrate’s intestine.

Our findings also showed that most of glucose storage is taking place in the intestinal muscle layer, probably for local use during contraction (39). Moreover, glycogen was also found in the enteric nervous system, probably in enteric glial cells (45),

Fig. 4. Glycogen (A) and lactate levels (B) and GK (C), low-Km HK (D), PK (E), FBPase (F), and LDH activities (G) in rainbow trout midgut pieces incubated in vitro for 6 h at 16°C in modified Cortland medium containing 0 (G0), 5 (G5), or 20 mM glucose (G20) alone (control) or with 10^{-8} M bovine insulin, 10^{-8} M human glucagon, 10^{-9} M rat glucagon-like peptide-1 (GLP-1), or 10^{-9} M rat C-peptide. Values are means ± SE of 10 (control) or 3 (treatments) independent experiments. *P < 0.05, significantly different from control within each glucose concentration. #P < 0.05, significantly different from groups incubated without glucose at the same treatment. †P < 0.05, significantly different from groups incubated with 5 mM glucose at the same treatment. When necessary, values were log transformed before statistical analysis.
which catabolize it to provide lactate. This lactate could be forwarded to neighboring neurons, a metabolic cooperation between astrocytes and neurons as found in the mammalian brain (4) and probably also in trout brain (43). GSase activities supporting the hypothesis that fish gut is a major glycolytic tissue in trout and that a key role in glucose homeostasis could be expected (22). The fact that lactate levels, LDH activity, and mRNA levels, as well as MCT-1 mRNA levels, increased only under hyperglycemia suggests that the enhanced glycolytic potential and lactate production are strongly glucose dependent. Our results for G6Pase activity and mRNA levels are consistent with the alternative mechanism of glucose transport in mammalian intestine. This involves glucose export to the bloodstream independently of GLUT2 but dependent on phosphorylation by HKs and further dephosphorylation by G6Pase (51). The increased activity and mRNA levels of GK in the trout gut could be explained by the necessity of phosphorylating high amounts of glucose for the above-mentioned mechanism.

The main contribution of small intestine in mammals to glucose homeostasis is through glucose production (22). In our study, G6Pase activity and mRNA levels seemed to be more related to glucose export into the bloodstream than to glucose production de novo. We found no regulation of FBPase activity by glucose, insulin, or amino acids. This is consistent with the lack of regulation in gluconeogenesis enzymes reported by Kirchner et al. (18) in trout fed diets with or without carbohydrates.

Table 2. Enzyme activities and metabolite levels in midgut pieces of rainbow trout incubated in vitro

<table>
<thead>
<tr>
<th>Glucose (5 mM)</th>
<th>Glucose (20 mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phlorizin</td>
<td>Cytochalasin B</td>
</tr>
<tr>
<td>Glucose</td>
<td>−287*</td>
</tr>
<tr>
<td>Glycogen</td>
<td>+184</td>
</tr>
<tr>
<td>Lactate</td>
<td>−436*</td>
</tr>
<tr>
<td>HK</td>
<td>n.d.</td>
</tr>
<tr>
<td>GK</td>
<td>n.d.</td>
</tr>
<tr>
<td>PK</td>
<td>−217*</td>
</tr>
<tr>
<td>LDH</td>
<td>−143*</td>
</tr>
<tr>
<td>FBPase</td>
<td>−132*</td>
</tr>
</tbody>
</table>

Values are means ± SE of 10 (control) or 3 (treatments) independent experiments in which enzyme activities and metabolite levels were measured in midgut pieces of rainbow trout incubated in vitro for 6 h at 16°C in modified Cortland medium containing 5 or 20 mM glucose alone (control) or with 1 mM phlorizin or 10 μM cytochalasin B. Data are expressed as percentages of increase (+) or decrease (−) compared with values in midgut pieces incubated with glucose alone. *P < 0.05, significantly different from control within each glucose concentration (Student’s t-test). When necessary, values were log-transformed before statistical analysis. HK, hexokinase; PK, pyruvate kinase; FBPase, fructose-1,6-bisphosphatase.

Our results suggest a minor effect of insulin on glucose metabolism in trout gut. The lack of changes in the activities of glucose phosphorylating enzymes suggests that the enhanced lactate production was not due to an increased glucose uptake from the lumen, consistent with the hypoglycemia experienced by insulin-injected fish (48). Whereas in mammals the intestine is an insulin-sensitive organ where insulin has a major role in glucose metabolism (8, 16), this seems not to be the case in trout, where only minor changes were noticed.

Glucose Metabolism in Trout Gut In Vitro

Because of the complexity of the intestine metabolism in vivo, several sets of in vitro experiments were carried out to elucidate the mechanisms underlying the metabolic changes observed. Even considering that metabolic rates are lower than those of transport rates (48), our findings showed that glucose metabolism in midgut pieces is strongly dependent on glucose transport, because specific glucose transport blockers elicited a decrease in glucose concentration, glucose phosphorylation, and oxidation and lactate production.

Because some of the results obtained with the insulin treatment could be due to insulin-induced hypoglycemia, we incubated midgut pieces in the presence of insulin and other glucose metabolism-related hormones, such as glucagon, GLP-1, and C-peptide (Fig. 4). In parallel with the increased glucose concentration in the medium, there was an enhancement of glucose phosphorylation and storage as glycogen, similar to the results obtained in vivo after oral glucose treatment. However, data about glycogen regulation in fish intestine are lacking, and its function remains to be elucidated. Although changes in HK and PK activities correlated with glucose levels in the medium, a saturated response was found at 20 mM glucose. Similar changes were found in lactate levels, showing that the excess of phosphorylated glucose not stored as glycogen was likely oxidized through glycolysis. These results support the hypothesis that, as in mammals (1), glucose might make a significant contribution to the energy requirement of the trout intestine.

Under our in vitro conditions, we found that most of the effects observed in vivo were reproduced (5 mM glucose alone and 5 mM glucose plus insulin), such as the lack of changes in glycogen levels and GK, PK, and FBPase activities and the increased LDH activity. The minor actions of insulin alone in fish intestine are not surprising (48) but contrast with those observed in mammals (54), suggesting that gut glucose metabolism in fish and mammals are differently regulated by insulin. The most relevant effect of glucagon in midgut was the marked glycojenolytic action when glucose was present in the medium. This agrees with the major effect described in fish hepatocytes (25) and is consistent with the presence of glucagon receptors in fish intestine (6). However, glycogen stores in trout gut are weak, suggesting that their physiological relevance might be related to a local use, as in skeletal muscle (21). The stimulation of GK activity by glucagon when glucose was in the medium was inconsistent with changes obtained in both glycogen levels and glycolytic potential. Also, the decreased glycolytic potential and higher FBPase activity after glucagon treatment agree with the scarce glucagon actions reported in the mammalian gut (19) and fish enterocytes (48). Our in vitro results with GLP-1 support information available concerning...
extrahepatic actions of GLP-1 (25), such as the presence of GLP-1 receptors (55) and the decreased glucose oxidation potential in fish enterocytes (48).

Moreover, most of the actions exerted by GLP-1 in trout are similar to those of glucagon, including increased glycogenolysis and gluconeogenesis and decreased glycolysis. This supports the idea that in fish gut, GLP-1 is overlapping glucagon actions, in a way similar to that observed in black bullhead enterocytes (48) and in fish liver (25). C-peptide actions in fish are almost unknown (26). In the present study,
the strongest metabolic effect (besides those found after glucose treatment) were those noticed after C-peptide incubations. Unlike in mammalian tissues, where C-peptide stimulates glucose transport and utilization (53), in the present study its main actions seem to be of anabolic nature (decreased glycolytic potential). Moreover, glycogen storage was also enhanced when glucose was present in the medium. Because C-peptide in mammals has been shown to interact with insulin receptors (14) and most of the results observed in the present study are similar to those of insulin (but even more potent), we can hypothesize that in fish intestine, C-peptide might exert insulin-like actions. However, more studies are needed to confirm this hypothesis and to explain why a synergistic effect was noticed.

An interesting result obtained in our in vivo studies was the positive correlation between plasma glucose levels and lactate production in intestine. However, because glucose oxidation could be enhanced in enterocytes as an artifact of tissue isolation in vitro, results must be interpreted with caution. Nevertheless, we have shown clearly that most of this lactate seems to be produced through the glycolytic pathway from glucose, which is relevant for a carnivorous fish like rainbow trout. In addition, some of the components involved in lactate metabolism, such as LDH and MCT-1, were characterized at the molecular level for the first time in fish. We have corroborated that the main substrate for this lactate production was glucose, because we found increased lactate production with glucose concentration in the medium and stable production when other substrates (like amino acids) were available. Because FBPase activity was not modified by glucose treatments, we suggest that this lactate is exported to the plasma in vivo to be utilized by other tissues (such as the liver), rather than being converted into glucose in situ, as in mammals (24).

The aim of the last set of in vitro incubations was to obtain more information about the putative ability of the trout gut to use amino acids as gluconeogenic substrates.
(Fig. 6). Despite the decreased FBPase activity (probably due to higher protein content in those treatments in which more amino acids were available, rather than to a decreased activity), the lack of regulation in vitro for FBPase activity could be related to its high activity, as in mammals, where this characteristic makes FBPase a nonlimiting enzyme for gluconeogenesis. However, no G6Pase or phosphoenolpyruvate carboxykinase activities were detected in our in vitro conditions.

Potential Glucosensing Capacity of Trout Gut

In mammalian intestine, sensing of luminal glucose can be accomplished by different cell types (38), including enterocytes, enteroendocrine cells (K and L), and components of the enteric nervous system. The glucosensing ability in mammalian intestine includes key components of the pancreatic glucosensing system (36, 44, 45) and is involved in functions like GLP-1 and glucose-dependent insulinotropic polypeptide (GIP) secretion. We have provided the first evidence in a nonmammalian vertebrate species for the presence in intestine of glucosensing components, including GLUT2, SGLT-1, GK, SUR-like, and Kir-like transcripts. Some of those proteins also have been related to glucosensing machinery in other trout tissues, such as brain regions and endocrine pancreas (40, 41). In mammals, the first step of intestinal glucosensing involves SGLT-1 and GLUT2 (7). In trout intestine, we found both GLUT2 and SGLT-1 transcripts, and SGLT-1 immunoreactivity was also detected in enterocytes and occasionally in enteroendocrine cells. In mammals, studies addressing the presence of GK in the gut are scarce (15, 36, 44), and its involvement in glucosensing is under debate in K and L cells, whereas it is clear in GLUTag cells (12). Although the involvement of GK in gut trout glucosensing remains to be demonstrated, the facts that GK gene expression and activity were enhanced in the midgut of hyperglycemic trout and that GK was localized in enteroendocrine cells constitute the first evidence for such a function in putative enteroendocrine cells of trout gut. In rodents and humans, KATP components (SUR1 and Kir6.2) were found in both L and K cells (31, 36, 44). These glucosensing components have been demonstrated to participate in GIP secretion in K cells (36) and to regulate the sensitivity of L cells to luminal glucose (44). As far as we are aware, this is the first time that KATP channel components have been found in fish intestine, although their involvement in glucosensing in
enteroendocrine cells of trout gut should be addressed in future experiments in which \( K_{\text{ATP}} \)-expressing cells could be isolated from the whole gut.

Although the involvement of enteric neurons in the reception of luminal glucose might be indirect (38), glucose has been shown to directly influence the activity of enteric neurons in mammals, although the mechanism underlying this ability remains under debate. Ma and Kirchgesner (20) detected (as in the present study) transcripts for GLUT2, GK, and \( K_{\text{ATP}} \) subunits in rat mucosa and enteric neurons. They presented immunolocalization for GK protein in endocrine cells, neurons, and varicose nerve fibers of the gut. The fact that in trout gut GK protein was also localized in equivalent cell types strongly supports the hypothesis of glucosensing neurons in trout gut. The presence of other components of the glucosensing system in enteric neurons should be addressed in future studies.

Conclusions and Perspectives

Glucose homeostasis in carnivorous fish species like rainbow trout has been studied for a long time, and the basis of the so-called “glucose intolerance” is still debated (28). Rainbow trout gut displays an important glucose metabolism based on an ability to store glucose as glycogen as well as a large capacity to oxidize glucose. This is surprising for a fish whose natural diet contains no more than 1% carbohydrates. That a large amount of glucose is oxidized to lactate supports the importance of glucose in gut metabolism and agrees with the metabolic characteristics of mammalian intestine. Supporting what other authors have suggested with dietary trials (18), we have confirmed the absence of regulation for gluconeogenesis in trout gut, which does not respond to hormone treatments or to changes in glucose, lactate, or amino acid levels, either in vivo or in vitro. On the other hand, we have corroborated the minor actions of insulin in trout gut, an insulin-sensitive tissue in mammals, whereas other hormones like GLP-1 and C-peptide have emerged as major hormonal regulators of fish gut glucose metabolism. We present preliminary evidence for the presence of a glucosensor system in trout gut, similar to that described in mammals (45) and in other trout tissues (40). The components involved in such putative function include SGLT-1, GK, and \( K_{\text{ATP}} \). GK and SGLT-1 have been localized in specific cells types similar to those involved in glucosensing in mammalian intestine, for example, those involved in GLP-1 or GIP components involved in such putative function include SGLT-1, GK, and \( K_{\text{ATP}} \). GK and SGLT-1 have been localized in specific cell types similar to those involved in glucosensing in mammalian intestine, for example, those involved in GLP-1 or GIP activities of jejunal carbohydrate-metabolizing enzymes in humans. AJP-Regul Integr Comp Physiol 299: R2275–R2283, 2007.

GK protein was also localized in equivalent cell types strongly supports the hypothesis of glucosensing neurons in trout gut. The presence of other components of the glucosensing system in enteric neurons should be addressed in future studies.

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Disclosures

No conflicts of interest, financial or otherwise, are declared by the author(s).

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