In vitro evidences for glucosensing capacity and mechanisms in hypothalamus, hindbrain and Brockmann bodies of rainbow trout

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ABBREVIATIONS

2-DG, 2-deoxy-D-glucose
BB, Brockmann bodies
HK-I, hexokinase I (EC. 2.7.1.11.)
GE, glucose-excited neurons
GI, glucose-inhibited neurons
GK, hexokinase IV or glucokinase (EC. 2.7.1.2.)
GLUT-2, glucose facilitative transporter type 2
GSase, glycogen synthase (EC. 2.4.1.11.)
ICV, intracerebroventricular
PK, pyruvate kinase (EC. 2.7.1.40.)
SUR-1, sulfonylurea receptor 1
ABSTRACT

We aimed to support *in vitro* the glucosensing capacity observed *in vivo* in rainbow trout hypothalamus, hindbrain, and Brockmann bodies (BB), and to obtain preliminary evidence of the mechanisms involved. The response of parameters involved in glucosensing capacity (HK, GK and PK activities, and glucose and glycogen levels) was assessed in these tissues incubated for 1 h with 2, 4 or 8 mM D-glucose alone (control) or with specific agonists/inhibitors of the steps involved in glucosensing capacity in mammals. These agents were a competitor for glucose phosphorylation (15 mM mannose), SUR-1 effectors (500 μM tolbutamide or diazoxide), glycolytic intermediates (15 mM glycerol, lactate or pyruvate), and inhibitors of glucose transport (10 μM cytochalasin B), glycolysis (20 mM 2-DG) and L-type calcium channel (1 μM nifedipine). Control incubations of the three tissues displayed increased glucose and glycogen levels and GK activities in response to increased medium glucose thus supporting our previous *in vivo* studies. Furthermore, critical components of the glucosensing mammalian machinery are apparently functioning in the three tissues. The responses in brain regions to all substances tested (except 2-DG and nifedipine) were similar to those observed in mammals suggesting a similar glucosensing machinery. In contrast, in BB only the effects of 2-DG, lactate, pyruvate, diazoxide and nifedipine were similar to those of mammalian β-cells, suggesting that some of the components of the piscine glucosensing model are different than those of mammals. Such differences may relate to the importance of amino acids rather than glucose signalling in the trout BB.

Key words: rainbow trout, glucosensor, hypothalamus, hindbrain, Brockmann bodies
INTRODUCTION

Teleost fish are traditionally considered relatively glucose-intolerant species (14,25,52). However, changes in circulating glucose levels elicit changes both at hormonal (insulin) and metabolic (liver GK activity and expression) levels indicative of the presence of the machinery needed to control blood glucose levels (25,33). In this sense, several recent studies provide evidence for glucosensing capacity in fish brain. This evidence is based upon i) experimentally-induced hyperglycemia leading to decreased food intake whereas increased food intake occurred after ICV treatment of rainbow trout with 2-deoxy-D-glucose (42); ii) parallel changes existing in rainbow trout brain between glycogen levels, GK activity and expression, and changes in plasma glucose levels (35); iii) rainbow trout re-fed for 7 days after 14 days of food deprivation displayed in both the hypothalamus and hindbrain increased GK activity and expression, and glycogen levels at the same time than plasma glucose levels increased (46); and, iv) glucose use in Myxocephalus scorpius brain increased under hypoglycemic conditions (21). Furthermore, in a recent study (34), after experimentally induced hyperglycemia and hypoglycemia in rainbow trout, we demonstrated in vivo in Brockmann bodies (BB, a distinct grouping of pancreatic islets near to the gall bladder) and in the hypothalamus and hindbrain a metabolic response that was distinct from that noted in other brain areas (midbrain, telencephalon) and the liver. The response to changes in plasma glucose was characterized by parallel changes in relative GLUT-2 expression, GK activity and expression, GSase activity, glycolytic potential, and levels of glycogen and glucose. Such changes are consistent with those reported in mammalian β-cells and glucose-excited (GE) neurons (20) supporting the presence of glucosensors in rainbow trout responsive to hyperglycemia and hypoglycemia.

The mechanisms involved in glucosensing are partially elucidated in mammalian β-cells and GE neurons (20,22): following a rise in plasma glyemia, glucose is transported across the membrane through a GLUT-2 carrier and metabolized by GK to glucose-6-phosphate that enters glycolysis and the Krebs cycle. The metabolism of glucose results in increased cellular ATP leading to closure of ATP-sensitive inward rectified K⁺ channels, membrane depolarization and Ca²⁺ influx. This scenario ultimately results in insulin release from β-cells or the increasing firing rate of GE neurons in the hypothalamus, medulla oblongata and mesencephalon.

There is evidence in fish for the presence of several of these same components including i) GK activity and expression, and GLUT-2 expression (31,32,34,46), ii) expression
of subunits of the K<sub>ATP</sub> channel such as kir (49,56) and SUR1 (56), and iii) presence of ATP-sensitive K<sup>+</sup> currents (30). No information exists as far as we are aware regarding the precise mechanisms by which these components respond when exposed to different glucose concentrations. This study, therefore, evaluates the effects of glucose concentrations and inhibitors/agonists on GK, low Km HK, and PK activities and glucose/glycogen content of the rainbow trout hypothalamus, hindbrain and BB incubated in vitro. These tissues were previously reported to have glucosensing capacity (34,46) and the various pharmacological inhibitors/agonists were previously used to characterize mammalian glucosensors. This inhibitors/agonists include an inhibitor of glucose transport (cytochalasin B), a competitor for glucose phosphorylation (mannose), an inhibitor of glycolysis (2-deoxy-D-glucose), a sulfonylurea receptor 1 agonist (tolbutamine), a sulfonylurea receptor 1 antagonist (diazoxide), an inhibitor of the L-type Ca<sup>2+</sup> channel (nifedipine), and several glycolytic intermediates (glycerol, lactate or pyruvate).

**MATERIALS AND METHODS**

**Fish**

Rainbow trout (*Oncorhynchus mykiss* Walbaum) were obtained from a local fish hatchery (Soutorredondo, Spain). Fish were maintained for 1 month in 100 litre tanks under laboratory conditions and a natural photoperiod in dechlorinated tap water at 14 °C. Fish mass was 98 ± 3 g. Fish were fed once daily (09.00 h) to satiety with commercial dry fish pellets (Dibaq-Diprotg SA, Segovia, Spain; proximate food analysis was 48% crude protein, 14% carbohydrates, 25% crude fat, and 11.5% ash; 20.2 MJ/kg of feed). The experiments described comply with the Guidelines of the European Union Council (86/609/EU), and of the Spanish Government (RD 1201/2005) for the use of animals in research.

**Experimental protocol**

Every morning of an experiment, fish were dipnetted from the tank, anaesthesized with MS-222 (50 mg·l<sup>−1</sup>) buffered to pH 7.4 with sodium bicarbonate, euthanized by decapitation, and weighed. The hypothalamus and hindbrain were removed and dissected as described previously (34,46); BB were dissected and cleaned from surrounding vessels, bile ducts, and connective tissue. Tissues were rinsed with modified Hanks’ medium (92.56 mM NaCl; 3.63 mM KCl, 2.81 mM NaHCO<sub>3</sub>, 0.85 mM CaCl<sub>2</sub>, 0.55 mM MgSO<sub>4</sub>, 0.4 mM KH<sub>2</sub>PO<sub>4</sub>, 0.23 mM Na<sub>2</sub>HPO<sub>4</sub>, 7.5 mM HEPES, 50 U·ml<sup>−1</sup> penicillin, and 50 mg·ml<sup>−1</sup>
streptomycin sulphate, pH 7.0; referred to a basal medium), sliced on chilled Petri dishes, and placed in a chilled Petri dish containing 100 ml of modified Hanks’ medium g\(^{-1}\) tissue that was continuously gassed with 0.5% CO\(_2\)/99.5% O\(_2\). To ensure adequate mass, tissues were combined from different fish resulting in pools of 3 hypothalami, 3-4 hindbrains and 3 BB. The tissue from each pool was finely minced and mixed and then placed in 48-well culture plates with 250 µl of modified Hanks’ medium (containing 20 mg of tissue) per well.

In preliminary experiments, tissues were incubated at different times ranging from 1 to 8 h at 15°C (data not shown). Tissues were viable throughout the whole time period though enzyme activities started to decrease from 2h onwards inducing us to choose 1h as optimal incubation period. In a second preliminary experiment, tissues were incubated at 15 °C for 1 h in modified Hanks’ medium containing 0, 0.1, 0.5, 1, 2, 4, 8, and 10 mM glucose to determine the linear range of response in parameters linked to changes in glucose concentration. The response was linear in the whole range (data not shown), and we selected a range close to plasma glucose levels in rainbow trout at concentrations from 2 to 8 mM glucose and these were selected for further experiments. The range of glucose concentration was also similar to that previously used by Ehrman et al. (7,8) for the assessment of somatostatin expression induced by glucose in isolated BB of rainbow trout incubated in vitro.

All subsequent experiments used freshly obtained tissues incubated in 48-well culture plates at 15°C for 1 h with 100 ml.g\(^{-1}\) modified Hanks’ medium that was gassed with 0.5% CO\(_2\)/99.5% O\(_2\). Control wells contained medium with 2, 4 or 8 mM D-glucose. Treated wells contained medium at the same glucose concentration and one of the selected agents related to glucosensing capacity in mammals. These included (final concentration) an inhibitor of glucose transport (10 µM cytochalasin B from Helminthosporium dematioideum), an inhibitor of glycolysis (20 mM 2-deoxy-D-glucose), a competitor for glucose phosphorylation (15 mM mannose), a sulfonyl urea receptor 1 agonist (500 µM tolbutamide), a sulfonyl urea receptor 1 antagonist (500 µM diazoxide), an inhibitor of L-type calcium channel (1 µM nifedipine), and several glycolytic intermediates (15 mM glycerol, lactate or pyruvate). All reagents were dissolved in modified Hanks’ medium, except for cytochalasin B, tolbutamide and nifedipine (all in 5% ethanol), and diazoxide (0.1 M NaOH). The concentrations of the different inhibitors used were selected based on those previously used in mammalian systems in vitro (10,23,27,28,29,36,51,54). No effects were observed due to the vehicle alone (data not shown). After 1h incubation tissues were quickly removed, frozen in liquid nitrogen, and stored at -80°C until assayed.
For each experiment, one set of 30 tissue pools was assessed (10 treatments x 3 glucose concentrations) for enzyme activities (GK, low Km HK, and PK) while a separate set of 30 tissue pools was used for the assay of tissue metabolites (glycogen and glucose levels). The number of independent experiments carried out for enzyme activities was three (N = 3) for treatments, and ten (N = 10) for controls, whereas a similar number of experiments (N = 3 for treatments and N = 10 for controls) was carried out to assess tissue metabolites.

Assessment of metabolite levels and enzyme activities

Tissue pieces used for the assessment of metabolite levels were homogenized immediately by ultrasonic disruption with 7.5 vols ice-cold 6% perchloric acid, then neutralized (using 1 mol·l⁻¹ potassium bicarbonate). The neutralized homogenate was centrifuged, and the supernatant used for assays. Tissue glycogen levels were assessed using the method of Keppler and Decker (16). Glucose obtained after glycogen breakdown (after subtracting free glucose levels) was determined with a commercial kit (Biomérieux, Spain). Limit of detection for glycogen and glucose levels was 1 nmol (glycosyl units or glucose).g⁻¹ wet mass.

Tissue pieces used to assess enzyme activities were homogenized by ultrasonic disruption with 9 vols ice-cold-buffer consisting of 50 mmol·l⁻¹ Tris (pH 7.6), 5 mmol·l⁻¹ EDTA, 2 mmol·l⁻¹ 1,4-dithiothreitol, and a protease inhibitor cocktail (Sigma Chemical Co., St. Louis, MO, USA; P-2714). The homogenate was centrifuged and the supernatant 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 by the increase or decrease in absorbance of NAD(P)H at 340 nm. The reactions were started by the addition of supernatant (15 μl) at a pre-established protein concentration, omitting the substrate in control wells (final volume 265-295 μl), and allowing the reactions to proceed at 20 °C for pre-established times (3-10 min). Enzyme activities are expressed in terms of mg protein. Protein was assayed in triplicate in homogenates using microplates according to the bicinchoninic acid method (40) with bovine serum albumin (Sigma, USA) as standard. Enzyme analyses were assessed at maximum rates by preliminary tests to determine optimal substrate concentrations. Low Km HK, GK, and PK activities were estimated as described previously (34,35,46). Limit of detection for enzyme activities was 0.1 mU.mg⁻¹ protein.

Statistics
Comparisons among groups were carried out using two-way ANOVA (SigmaStat; SPSS Inc., Chicago, IL) with glucose concentration (2, 4, and 8 mM) and treatment as the main factors. When necessary data were log transformed to fulfill the conditions of the analysis of variance. Post-hoc comparisons were made using a Student-Newman-Keuls test, and differences were considered statistically significant at $P<0.05$.

RESULTS

GK activity

In hypothalamus (Fig. 1A) control activities increased with glucose concentration. Diazoxide increased GK activity at 2 and 4 mM glucose but decreased at 8 mM glucose. Cytochalasin B treatment only decreased activities at 8 mM glucose. 2-DG, mannose, glycerol, tolbutamide or nifedipine addition resulted in higher GK activities but in the absence of a trend to increase with glucose concentration.

In hindbrain (Fig. 1B) control activities increased with glucose concentration in the medium. Increased GK activity when glucose concentration in the medium increased was also noted for hindbrains treated with mannose, nifedipine and pyruvate. Under nifedipine and lactate treatments, the activity was higher than the control only when the medium contained 2 mM glucose. Lactate incubation reduced GK activities compared with the control at the two higher glucose concentrations assessed. A decrease was also found under 2-DG (8 mM glucose concentration) and glycerol (2 and 4 mM glucose concentration) treatments. In contrast, a rise in GK activity occurred at 2 mM glucose with diazoxide in the medium.

In Brockmann bodies (Fig. 1C) activity in control tissues showed a trend to increase with medium glucose concentrations. A similar trend was observed with diazoxide, tolbutamide, lactate and 2-DG treatments. Under mannose treatment, GK activity was higher than the control only at 2 mM glucose. BB incubated with nifedipine or pyruvate showed a peak of GK activity at 4 mM glucose, but the nifedipine treatment always demonstrated lower activities than the control.

Low Km HK activity

In hypothalamus (Fig. 2A) activities were unaffected in the controls by glucose concentration. Both cytochalasin B and lactate generally decreased activities compared with controls with the lactate effect exaggerated at the highest glucose concentration. Glycerol and
mannose treatments generally increased activities compared with the controls though not at 2 mM glucose for glycerol and 8 mM glucose for mannose.

In hindbrain (Fig. 2B) no differences were noted in controls incubated with the 3 glucose concentrations used. Incubating with glycerol, tolbutamide or nifedipine increased low Km HK activities only at 2 mM glucose; pyruvate treatment increased activities at 4 mM glucose. 2-DG and diazoxide treatments showed peak activities at 4 mM glucose. Cytochalasin B reduced low Km HK activities compared with the controls at 2 mM glucose whereas lactate did it at the three glucose concentrations assessed. The opposite was observed when the treatment was mannose.

In Brockmann bodies (Fig. 2C) no significant differences in the control group were observed with respect to medium glucose concentrations. Pyruvate treatment decreased activities compared with the control at all glucose concentrations while glycerol incubation resulted in lower activities than that of the control at both 2 and 4 mM glucose. Diazoxide treatment decreased activities only at 2 mM and nifedipine at 2 and 8 mM glucose. Tolbutamide treatments showed a clear correlation with medium glucose, being lower than controls at 2 mM and higher at 8 mM.

Glucose concentration

In hypothalamus (Fig. 3A) levels clearly increased in controls as glucose concentration in the media increased; this effect was countered in the presence of both 2-DG and cytochalasin B. The increase in glucose levels as glucose concentration in the media increase was not affected by the other treatments.

In hindbrain (Fig. 3B) levels were directly related to glucose concentration in the control medium. The increase in glucose levels as glucose concentration in the media increase was not affected by mannose, diazoxide, glycerol, tolbutamide, nifedipine, pyruvate, and lactate treatments. When the hindbrains were incubated with 2-DG, glucose levels increased and the correlation with medium glucose disappeared. Cytochalasin B treatment generally decreased glucose levels compared with controls, although at 8 mM glucose were above those at 2 and 4 mM. Glucose levels in pieces incubated with nifedipine, pyruvate or lactate showed lower values than those of controls but only when glucose concentration in the medium was 8 mM.

In Brockmann bodies (Fig. 3C) glucose levels in controls demonstrated a clear increase with glucose concentration in the medium. A similar increase with glucose concentration was observed for BB incubated with cytochalasin B, mannose, diazoxide,
tolbutamide, nifedipine, pyruvate, and lactate, although for both cytochalasin B and lactate these values were generally lower than those of the control. Glycerol incubation increased glucose levels only at 4 mM glucose.

**Glycogen levels**

In hypothalamus (Fig. 4A) levels in controls increased with glucose concentration in the medium. 2-DG increased glycogen content compared with controls also producing the dissappearance of the increased levels response of controls when glucose concentration increased in the media. The increase in glycogen levels when glucose concentration increased in the media was not affected by the other agents, except for lactate at 4 mM and diazoxide at 8 mM that were significantly depressed compared with both the controls and the 2 mM glucose.

Levels of glycogen in hindbrain (Fig. 4B) generally increased with glucose concentration in the medium in controls. A similar increase was noticed for hindbrains incubated with glycerol. Values of glycogen under 2-DG treatment were higher than those of controls at 2 and 4 mM glucose, but this increase was overcome at 8 mM glucose. Incubations with cytochalasin B decreased glycogen levels at 8 mM glucose whereas mannose treatment also induced a decrease at 2 mM glucose. Under lactate and mannose treatments glycogen levels were similar to those of the control group but showed a significant decrease at 2 mM compared with the other glucose concentrations. Glycerol treatment showed a similar effect but without the fall at 2 mM glucose. Pyruvate and diazoxide showed inverse trends with pyruvate showing higher glycogen values at 4 mM compared with 2 and 8 mM glucose, while diazoxide showed the opposed pattern.

In BB (Fig. 4C) glycogen levels of controls displayed a trend to increase with medium glucose concentrations. Similar trends were noted for BB incubated with mannose, glycerol, diazoxide, and tolbutamide. With cytochalasin B, pyruvate or lactate treatments the levels were lower than controls (even undetectable at least with cytochalasin B at 2 mM glucose). Under 2-DG treatment, no significant differences were noted at 2 mM whereas a 10- and 2-fold increase was found at 4 and 8 mM glucose, respectively. A strong correlation was apparent between BB glycogen levels and medium glucose concentrations with glycerol treatment.
PK activity

In hypothalamus (Fig. 5A) levels of controls were lower at 8 mM glucose than at 2 or 4 mM. Activities in controls were higher than in hypothalamus treated with mannose (except at 8 mM glucose), but lower than those treated with 2-DG (except at 4 mM glucose), glycerol and diazoxide. Under pyruvate treatment activity shows a peak at 4 mM whereas with nifedipine and lactate treatment the increase appears at 8 mM.

In hindbrain (Fig. 5B) no differences were observed in controls with respect to medium glucose concentration and no PK activity was measurable when incubations included diazoxide, nifedipine or tolbutamide. 2-DG treatment resulted in higher values than controls at 4 and 8 mM of glucose while cytochalasin B reduced activities at 4 mM glucose. PK activities increased with glycerol treatment at 4 mM glucose, but were lower than controls at 2 and 8 mM glucose. Pyruvate treatment increased activities at 4 and 8 mM but no changes were observed at 2 mM glucose. PK activity showed an inverse correlation with glucose concentration in the medium when the hindbrains were incubated with lactate.

In BB (Fig. 5C) PK activities trended to increase with medium glucose in the controls. The same trend was found under pyruvate treatment, but with lower activities than the control. No PK activity was detectable with 2-DG treatment at 2 mM medium glucose whereas decreased activities occurred at 4 and 8 mM. With both cytochalasin B and nifedipine incubation significant decreases in PK activities were apparent at 8 mM glucose. The same fall in PK activity at 8 mM glucose concentration was observed with the other treatments, but accompanied by increased activity at 2 mM (mannose or tolbutamide); decreases at 2 mM (lactate) or 4 mM (glycerol or diazoxide).

DISCUSSION

The physiological mechanisms involved in glucose homeostasis involve the coordinated function of several organs, and key to this control is the presence of tissue glucosensors (50). Despite the relative glucose intolerance of fish (14,25), plasma glucose levels also fluctuate in fish (12,35) and it is proposed that control of glucose levels exist at least in those tissues that rely on glucose as primary fuel such as the brain (41). Accordingly, previous evidence supports the existence of glucosensors in rainbow trout BB (34) that may be involved in the increased insulin release reported under hyperglycemic conditions (11). Evidence also exists for glucosensors in rainbow trout hypothalamus and hindbrain (34,46)
where they may be involved in the orexigenic and anorexigenic responses observed under either hypoglycemic or hyperglycemic conditions (42).

This study provides *in vitro* support for our previous *in vivo* studies by incubating the presumed glucosensing tissues with concentrations of glucose ranging from 2 to 8 mM. This range was chosen based on previous *in vitro* experiments with trout BB that showed increased expression of somatostatin with glucose (7,8). Control incubations of the three tissues assessed in our study clearly showed increased response to increased media glucose for those parameters expected to change with glucose concentration, such as glucose and glycogen levels, and GK activity. No changes directly related to glucose concentration were observed for low Km HK and PK activities in brain regions, in agreement with a similar lack of response observed *in vivo* (34) and reinforces the specificity of the glucosensing response of those brain regions. In contrast, PK activity in BB increased with glucose concentration in a manner similar to that observed in mammals where exposure to elevated glucose levels in β-cells caused induction of glycolytic enzymes including 6-phosphofructo 1-kinase and PK (37). These *in vitro* results support those previously obtained *in vivo* with trout (34,46) and provide further evidence for the presence of glucosensors in these three tissues.

A typical mammalian glucose sensor is characterized by the presence in the following order of GLUT-2, GK, ATP/ADP-controlled K⁺ channel, and voltage-gated L-type Ca²⁺ channel (22). Despite several reports in fish for the presence of these components (34,56), no information is available regarding their behavior to changes in glucose levels in presumptive glucosensing tissues. The following paragraphs will place in context our results using agents known to affect components of the glucosensing machinery of mammalian β-cells and GE neurons to support the existence of similar machinery in fish glucosensing tissues.

*Effects of cytochalasin B*

Inhibitors of glucose transport block effects of increased glucose levels in mammalian glucosensing systems (39,55). Cytochalasin B is a well-recognized blocker of glucose transport through GLUT carriers in mammals and in several fish tissues (43,44). Cytochalasin B treatment in this study offset the increase in glucose but not glycogen content noted as medium glucose increased in the controls of both hypothalamus and hindbrain. GK activities did not parallel these changes except in hypothalamus incubated with 8 mM glucose. These effects are similar to those previously described after treatment with glucose transport blockers in mammalian GE and glucose-inhibited (GI) neurons (39,55). In contrast, no significant effects of cytochalasin B were noted in BB regarding either GK activity or
Effects of mannose

The hexose mannose is metabolized by GK with a similar Km to that of glucose (53); therefore, mannose is able to mimic the effects of glucose on GK activity (10) stimulating glucosensing systems (39,54). Mammalian β-cells incubated with glucose and mannose increased GK protein level when compared with those incubated with glucose alone (10). In the present study, mannose was added at 15 mM and GK activities in BB increased compared with those at 2 mM glucose but decreased at 4 and 8 mM glucose; these results support a differential utilization of mannose by the trout compared with the mammalian BB GK enzyme. Mannose generally increased trout brain GK activities compared with controls at all medium glucose levels. In contrast, glycogen levels increased in hypothalamus and hindbrain (not in BB) supporting synthesis of glycogen from glucose 6-phosphate derived from mannose (5). Finally, it is very interesting that glycolytic capacity (as assessed by PK activities) is differentially affected by mannose treatment in the three tissues assessed, decreasing in hypothalamus and hindbrain but without affect in the BB. This may relate to a lower glycolytic potential in BB compared with the two brain regions. Taken together mannose addition appears to mimic the response of the tissues assessed to increased glucose levels in a way similar to that described in mammals.

Effects of 2-deoxy-D-glucose

2-DG is a glucose analog that can be phosphorylated by low Km HK/GK at a higher Km than that of glucose (53) but cannot be further metabolized resulting in an inhibition of glycolysis (28). 2-DG incubation clearly affected glucose levels in the present study in all three tissues assessed although it increased levels in both hindbrain and BB at 2 and 4 mM glucose (not at 8 mM) while decreasing levels in the hypothalamus. Moreover, the increase observed in controls as medium glucose increased, generally dissappeared with 2-DG-treatment. Similarly, GK activities showed no major differences in the three tissues vs. control fish though the increased activity observed with medium glucose in controls dissappeared in 2-DG-treated tissues. The absence of changes in GK activities to 2-DG treatment is in agreement with similar results obtained in mammalian β-cells (10,26) whereas an inhibition of GK activity has been reported in mammalian GE neurons after 2-DG treatment (54,55). 2-DG treatment induced a general increase in glycogen levels in the three tissues assessed and
in particular hypothalamus and BB similar to that observed in mammalian liver (3). It is apparent that 2-DG treatment of trout BB is effective as in mammalian pancreas in blocking the responses assessed to increased levels of glucose but this not happened in brain.

**Effects of glycolytic intermediates**

In mammalian glucosensing systems glycolytic intermediates including galactose, glyceraldehyde, glycerol and lactate generally mimic glucose effects whereas pyruvate does not (39,54,55). The addition of glycerol to trout tissues in the present study increased GK activities and glucose levels in both brain regions but not in BB. This behavior in fish tissues does not coincide with the mammalian model where increased GK activity and glucose levels occur after glycerol treatment in both brain regions (54,55) and β-cells (39).

Lactate inhibits the effects of increased glucose levels in mammalian GE neurons (1). Under low glucose concentrations, mammalian glucosensing neurons may potentially use astrocyte-derived lactate as an alternate regulator of firing rate (4,17). This is different from β-cells that are unable to use lactate to increase insulin secretion (15). The addition of medium lactate in the present study decreased GK activities as medium glucose concentrations increased which is the opposite response to that observed in the controls. Given that this response occurred in the hypothalamus and hindbrain but not in BB allows us to hypothesize that lactate has a similar metabolic role in brain as that described in mammals (17). Since lactate is known to reduce glucose sensitivity in GI neurons of suckling rats (48), and brain of suckling rats can use lactate as fuel in a way similar to that of fish brain (45), the decrease observed in hypothalamus and hindbrain GK activities may also support a similar capacity for lactate in glucosensing brain regions of fish. Therefore, we may hypothesize that lactate under high glucose concentration induces an inhibitory effect on the glucosensing machinery whereas under low glucose concentrations lactate would be used as an alternative fuel thus maintaining the potential of glucosensing. Moreover, if the BB does not use lactate as a fuel in fish as in the mammalian pancreas (15), no effects of lactate would be expected. Glucose levels in lactate-treated tissues increased with medium glucose concentrations in a similar manner to controls, whereas such an increase did not occur with glycogen levels. It is therefore apparent that lactate is not interfering with glucose entry into the cell but inhibiting glucose metabolism through GK resulting in less glycogen synthesis. Moreover, an inhibition of glycolytic capacity was evident in hindbrain and BB but not in hypothalamus of lactate-treated tissues.
Pyruvate, the end product of aerobic glycolysis does not stimulate mammalian GE neurons (54,55) or β-cells (9,19). Pyruvate did not affect in the present study changes observed in GK activity or glucose/glycogen levels of controls when incubated with increased medium glucose concentrations in hypothalamus and hindbrain and generally not in BB. This supports that in fish as in mammals pyruvate is not mimicking glucose effects in glucosensitive areas.

**Effectors of the K<sub>ATP</sub> channel**

The K<sub>ATP</sub> channel is an inwardly rectified K<sup>+</sup> channel involved in glucosensing activity in mammalian β-cells and GE neurons. It is an octomeric complex consisting of four sulfonylurea receptors (SUR) and four K<sup>+</sup> channel subunits (38). In glucosensing cells the K<sub>ATP</sub> channel is closed by increased cellular ATP/ADP ratios mimicking the effects of increased plasma glucose; sulfonylureas binding to the SUR subunits bypasses this ATP/ADP signal (1,6,38,39). The incubation with a sulfonylurea like tolbutamide in the present study elicited increased GK activity in the trout hypothalamus and hindbrain but not in BB when compared with controls. Moreover, the dose-dependent increase observed in control activities with respect to medium glucose levels dissappeared in tolbutamide-treated tissues. Mammalian GI neurons do not use K<sub>ATP</sub> channels to sense glucose (15) and a similar situation may exist in BB at low medium glucose concentrations (2 mM). In contrast, tolbutamide did not affect control glucose/glycogen levels that increased with medium glucose levels. Sulfonylurea treatment of mammalian heart and liver is reported to mobilize glycogen stores and increase glycolytic flux (18). In contrast, tolbutamide treatment of trout tissues did not affect significantly PK activities whereas glycogen levels did fall but only in hypothalamus incubated with 8 mM glucose. Therefore, tolbutamide is apparently enhancing the effects of increased glucose levels on trout GK activities in brain regions but not in BB.

Diazoxide opens K<sub>ATP</sub> channels via the SUR1 subunit thus inhibiting insulin release in mammalian β-cells (39) and decreasing firing rate in GE neurons (47). Therefore, diazoxide should counter the effects observed in control incubations to increasing medium glucose. This prediction is satisfied in trout hypothalamus where GK activities decreased as medium glucose increased, and in BB where decreased activity occurred at lower glucose concentrations. The effects on hypothalamus are interesting as they are similar to those reported in livers of hyperglycemic rats (comparable to the incubations with 8 mM glucose in our experiments) where a decreased GK activity occurred with diazoxide treatment when no change was observed in normoglycemic rats (2). The different behaviours noted for the trout
brain regions regarding GK activities may be related to the expression of SUR1; in mammals not all GE neurons express SUR1 and only those that do express this receptor are sensitive to diazoxide (54). Accordingly, trout hypothalamus may contain a higher proportion of SUR1 receptors than the hindbrain.

Interestingly, diazoxide treatment increased PK activities above those of controls in the trout hypothalamus whereas decreased activities were noted in BB and no activities were observed in the hindbrain. An increased glycolytic capacity is reported in the liver of hyperglycemic rats treated with diazoxide (2). In the trout this drug is antagonizing the effects of increased glucose levels on PK activities in hindbrain and BB leading to a decrease whereas a glycolytic increase occurred in the hypothalamus only at 8 mM glucose, a concentration comparable to hyperglycemic conditions.

**Effects of nifedipine**

The opening of L-type Ca\(^{2+}\) channels is involved in glucosensing signaling in both β-cells and GE neurons (20,22). That type of calcium channel can be blocked by dihydropiridines including nifedipine (13) resulting in an inhibition of the glucosensing response produced by increased glucose levels (54,55). The only significant effect of nifedipine incubation of trout tissues was a GK activity increase in the two brain regions and a decrease in BB. Moreover, the increase observed in GK activities of controls with increased medium glucose was countered by nifedipine treatment. Thus, the trout BB responds to nifedipine as in mammals, but the brain does not. Moreover, an increase in intracellular [Ca\(^{2+}\)] can trigger mobilization of brain glycogen, and this effect is blocked by nifedipine (13). In contrast, in the fish no major changes occurred in glycogen levels suggesting that nifedipine cannot block as effectively glycogen metabolism.

**Conclusion/Perspectives**

Our previous *in vivo* studies (34) provided evidence of the existence of glucosensors in hypothalamus, hindbrain and Brockmann bodies of rainbow trout responding to changes in glucose levels with changes in metabolic parameters similar to those previously demonstrated in mammalian glucosensing systems. This study examined these same tissues incubated *in vitro* and demonstrated for the first time their response to increased medium glucose consistent with a glucosensing capacity. The use of specific inhibitors and agonists further support that critical components of the glucosensing machinery demonstrated in mammals are apparently present in rainbow trout hypothalamus, hindbrain and BB. Most components of the
glucosensing machinery of the mammalian brain are present in the trout brain although not all as neither 2-DG nor nifedipine acted in the fish as in mammals. The situation in the BB was quite different since several of the effects were different (even opposite in some cases) to those reported in mammalian β-cells including for the effects of cytochalasin B, mannose, glycerol and tolbutamide. In contrast, the effects of 2-DG, lactate, pyruvate, diazoxide or nifedipine were similar in trout BB to those of the mammalian β-cell suggesting that at least some components of the mammalian system may be working in this piscine model. Future studies are necessary to elucidate more clearly why such differences exist in the glucosensing mechanisms between the brain and the BB of trout. These differences may relate to the fact that glucose constitutes a small portion of the normal fish diet (14,52) and the fact that amino acids are better insulin secretagogues in fish than glucose (24). Possibly, BB function is more dependent than the brain regions on other metabolic signals such as amino acids requiring adjustments in the BB sensing system. In contrast, the glucosensing capacity of the brain regions is more similar to that of mammals in agreement with the role of glucose as a metabolic fuel for brain function in fish (41). However, considering the responses observed after lactate treatment in brain regions, this fuel can be also considered as a metabolic signal in brain glucosensing systems and this deserves further attention. Furthermore, studies are needed to assess the precise links between the activation of the glucosensing system in all tissues assessed and the physiological responses in brain (orexigenic and anorexigenic responses) and BB (insulin release). Finally, the present data in rainbow trout do not support that the relative glucose intolerance of carnivorous fish could be attributed to the lack of glucose sensing capacity, which therefore may rely in other mechanisms occurring once glucosensing mechanisms elicited their physiological response.

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REFERENCES


Figure legends

Fig. 1. Glucokinase activities in hypothalamus (A), hindbrain (B), and Brockmann bodies (C) of rainbow trout incubated in vitro for 1 h at 15 °C in modified Hanks’ medium containing 2, 4 or 8 mM glucose alone (control) or with 20 mM 2-deoxy-D-glucose, 10 µM cytochalasin B, 15 mM mannose, 15 mM glycerol, 500 µM diazoxide, 500 µM tolbutamide, 1 µM nifedipine, 15 mM pyruvate or 15 mM lactate. Each value is the mean + S.E.M. of ten (control) or 3 (treatments) independent experiments carried out with pools of tissues from 3-4 different fish (30 pools per experiment, 10 treatments x 3 glucose concentrations). Different letters indicate significant differences (P<0.05) among treatments within each glucose concentration. #, Significantly different from groups incubated with 2 mM glucose at the same treatment (P<0.05); §, significantly different from groups incubated with 4 mM glucose at the same treatment (P<0.05). When necessary values were log transformed prior to statistical analysis.

Fig. 2. Low Km hexokinase activities in hypothalamus (A), hindbrain (B), and Brockmann bodies (C) of rainbow trout incubated in vitro for 1 h at 15 °C in modified Hanks’ medium containing 2, 4 or 8 mM glucose alone (control) or with 20 mM 2-deoxy-D-glucose, 10 µM cytochalasin B, 15 mM mannose, 15 mM glycerol, 500 µM diazoxide, 500 µM tolbutamide, 1 µM nifedipine, 15 mM pyruvate or 15 mM lactate. Each value is the mean + S.E.M. of ten (control) or 3 (treatments) independent experiments carried out with pools of tissues from 3-4 different fish (30 pools per experiment, 10 treatments x 3 glucose concentrations). Further details as in legend to Fig. 1.

Fig. 3. Glucose levels in hypothalamus (A), hindbrain (B), and Brockmann bodies (C) of rainbow trout incubated in vitro for 1 h at 15 °C in modified Hanks’ medium containing 2, 4 or 8 mM glucose alone (control) or with 20 mM 2-deoxy-D-glucose, 10 µM cytochalasin B, 15 mM mannose, 15 mM glycerol, 500 µM diazoxide, 500 µM tolbutamide, 1 µM nifedipine, 15 mM pyruvate or 15 mM lactate. Each value is the mean + S.E.M. of ten (control) or 3 (treatments) independent experiments carried out with pools of tissues from 3-4 different fish (30 pools per experiment, 10 treatments x 3 glucose concentrations). Values not shown are below the limit of detection (1 nmol glycosyl units.g⁻¹ wet mass). Further details as in legend to Fig. 1.

Fig. 4. Glycogen levels in hypothalamus (A), hindbrain (B), and Brockmann bodies (C) of rainbow trout incubated in vitro for 1 h at 15 °C in modified Hanks’ medium containing 2, 4 or 8 mM glucose alone (control) or with 20 mM 2-deoxy-D-glucose, 10 µM cytochalasin B, 15 mM mannose, 15 mM glycerol, 500 µM diazoxide, 500 µM tolbutamide, 1 µM nifedipine, 15 mM pyruvate or 15 mM lactate. Each value is the mean + S.E.M. of ten (control) or 3 (treatments) independent experiments carried out with pools of tissues from 3-4 different fish (30 pools per experiment, 10 treatments x 3 glucose concentrations). Values not shown are below the limit of detection (1 nmol glycosyl units.g⁻¹ wet mass). Further details as in legend to Fig. 1.

Fig. 5. Pyruvate kinase activities in hypothalamus (A), hindbrain (B), and Brockmann bodies (C) of rainbow trout incubated in vitro for 1 h at 15 °C in modified Hanks’ medium containing 2, 4 or 8 mM glucose alone (control) or with 20 mM 2-deoxy-D-glucose, 10 µM cytochalasin B, 15 mM mannose, 15 mM glycerol, 500 µM diazoxide, 500 µM tolbutamide, 1 µM nifedipine, 15 mM pyruvate or 15 mM lactate. Each value is the mean + S.E.M. of ten (control) or 3 (treatments) independent experiments carried out with pools of tissues from 3-4 different fish (30 pools per experiment, 10 treatments x 3 glucose concentrations). Values not
shown are below the limit of detection (1 mU.mg⁻¹ protein). Further details as in legend to Fig. 1.
Fig. 1. Polakof et al.
Fig. 2. Polakof et al.
Fig. 4. Polakof et al.
Fig. 5. Polakof et al.