ACTH-stimulated cortisol release from head kidney of rainbow trout is modulated by glucose concentration

Marta Conde-Sieira¹, Rosa Alvarez², Marcos A. López-Patiño¹, Jesús M. Míguez¹, Gert Flik³, and José L. Soengas¹

Laboratorios de ¹Fisioloxía Animal and ²Bioloxía Celular, Departamento de Bioloxía Funcional e Ciencias da Saúde, Facultade de Bioloxía, Universidade de Vigo, Spain.
³Department of Animal Physiology, Institute for Water and Wetland Research, Faculty of Science, Radboud University Nijmegen, Nijmegen, The Netherlands

Running title: glucose affects cortisol release in trout

Corresponding author:

Prof. José L Soengas
Laboratorio de Fisioloxía Animal
Facultade de Bioloxía
Edificio de Ciencias Experimentais
Universidade de Vigo
E-36310 Vigo
Spain
Tel. +34-986 812 564
Fax +34-986 812 556
E-mail jsoengas@uvigo.es
Abbreviations

3βHSD, 3β-hydroxysteroid dehydrogenase
11βH, 11β-hydroxylase
ACTH, adrenocorticotropic hormone
CRF, corticotrophin releasing factor
GK, hexokinase IV or glucokinase (EC. 2.7.1.2.)
GLUT-2, glucose facilitative transporter type 2
Gnat3, gustducin
GSase, glycogen synthase (EC. 2.4.1.11.)
K\textsubscript{ATP}, ATP-dependent inward rectifier potassium channel
Kir6.x-like, inward rectifier K\textsuperscript{+} channel pore type 6.x-like
LXR, liver X receptor
P450scc, cytochrome P450 cholesterol side chain cleavage
PK, pyruvate kinase (EC. 2.7.1.40.)
StAR, steroidogenic acute regulatory protein
SGLT-1, sodium-glucose linked transporter type 1
SUR-like, sulfonylurea receptor-like
TH, tyrosine hydroxylase
Summary

To assess the hypothesis of cortisol release in rainbow trout being modulated by glucose levels, we first evaluated cortisol release (basal and ACTH-regulated) by head kidney tissue superfused with medium reflecting hypo-, normo- or hyperglycaemic conditions. Next, cortisol release from head kidney fragments in static incubations was assessed in parallel with changes in parameters related to cortisol synthesis (mRNA abundance of StAR, P450scC, 3βHSD, and 11βH) and the GK-mediated glucosensing mechanism (levels of glycogen and glucose, activities of GK, GSase, and PK, and mRNA levels of GK, GLUT-2, Kir6.x-like, and SUR-like). We then evaluated the effects of two inhibitors of glucose transport cytochalasin B and phlorizin on cortisol production and glucosensing mechanisms. The ACTH-induced release of cortisol proved to be modulated by medium glucose concentration in a way that increased release occurs under high glucose levels, and decreased ACTH-stimulated cortisol release occurs when glucose transport was inhibited by cytochalasin B. The release of cortisol can be associated with increased synthesis since enhanced mRNA abundance of genes related to cortisol synthesis was also noted in high glucose medium. Specific GK-immunoreactivity in the cortisol producing cells (not in chromaffin cells) further substantiates GK-mediated glucosensing in cortisol production. In contrast, no changes compatible with those of glucose levels and cortisol release/synthesis in the presence of ACTH were noted for any other putative glucosensor mechanisms based on LXR, SGLT-1 or Gnat3. The results combined are the first evidence for a mechanism in fish linking synthesis and release of a non-pancreatic hormone like cortisol with circulating glucose levels. The relationship was evident for the regulated (ACTH-dependent) pathway and this suggests that under acute stress conditions glucose is important for the regulation of cortisol synthesis and release.

Key words: rainbow trout, head kidney, cortisol, glucose
Introduction

Cortisol is the main steroid produced in fish head kidney, which harbors the interrenal cells, homologues of the mammalian adrenal zona fasciculata cells. In fish, cortisol plays an important role in energy homeostasis, growth and osmoregulation (Mommsen et al., 1999). The main secretagogue for cortisol is adrenocorticotrophic hormone (ACTH) released from the anterior pituitary; ACTH release, in turn, is controlled by corticotrophin releasing factor (CRF) produced by the hypothalamus (Wendelaar Bonga, 1997). The main proteins involved in the regulation of the corticosteroidogenic pathway (Payne and Hales, 2004) comprise steroidogenic acute regulatory (StAR) protein, cytochrome P450 cholesterol side chain cleavage (P450scc), 3β-hydroxysteroid dehydrogenase (3βHSD), and 11β-hydroxylase (11βH).

We demonstrated in previous studies in rainbow trout hypothalamus, hindbrain and Brockmann bodies the existence of a glucosensor mechanism dependent on glucokinase (GK), glucose facilitative transporter type 2 (GLUT-2), and ATP-dependent inward rectifier potassium channel (K_{ATP}) (see review Polakof et al., 2011). The latter glucosensor system is adjusted under stress conditions, like those associated with high stocking density, resulting in its inability to respond to changes in circulating glucose levels (Conde-Sieira et al., 2010a,b). The adjustment of glucosensor in central areas (hypothalamus and hindbrain) under stress conditions is apparently mediated by CRF since treatment with this neuropeptide affects the sensitivity of glucosensing mechanism in a way similar to that observed under stress conditions (Conde-Sieira et al., 2011). In those studies we observed a very complex interaction among cortisol levels, glycaemia, and stress (Conde-Sieira et al., 2010a,b). Therefore, we hypothesize that cortisol synthesis and release in interrenal tissue may be modulated by circulating glucose levels, i.e. that some type of glucosensor mechanism is operational in interrenal cells of rainbow trout, in a way similar to that characterized in pancreatic β-cells where the glucosensor system is related to insulin release. In other studies carried out in rainbow trout, plasma cortisol levels decreased in situations known to produce hypoglycaemia such as food deprivation (Pottinger et al., 2003; Polakof et al., 2007a) whereas plasma cortisol levels increased under hyperglycaemic conditions such as post-feeding (Bry 1982; Holloway et al., 1994) or exercise (Nielsen et al., 1994). A recent study carried out in zebrafish provided direct evidence for a relationship between cortisol levels and glycaemia since levels of whole body cortisol were dependent on glucose (Powers et al., 2010). In mammals, cortisol secretion from adrenal cortex has been also hypothesized to be related to
glycaemic conditions (Andrews et al., 2002) though evidence obtained to date is not conclusive since cortisol levels do not change under hyperglycaemic conditions alone (Andrews et al., 2002) necessitating an additional stress to increase (Kirschbaum et al., 1997).

To assess the hypothesis of glucose levels modulating cortisol release in rainbow trout, we evaluated in a first experiment changes in cortisol release (in the absence or presence of ACTH) when head kidneys were superfused with media mimicking conditions of hypo-, normo- and hyperglycaemic conditions (2, 4, and 8 mM glucose, respectively) as previously described (Polakof et al., 2007b). Since changes were noted, we carried out a second experiment in which cortisol release from head kidney slices incubated in vitro was evaluated in parallel with changes in parameters related to cortisol synthesis (mRNA abundance of StAR, P450scc, 3βHSD, and 11βH) and to the glucosensor characterized in rainbow trout (Polakof et al., 2011), including levels of glycogen and glucose, activities of GK, glycogen synthase (GSase) and pyruvate kinase (PK), and mRNA levels of GK, GLUT-2, inward rectifier K\(^+\) channel pore type 6.-like (Kir6.x-like), and sulfonylurea receptor-like (SUR-like). Moreover, we also evaluated in the same experiment the presence of components of any other putative glucose sensor mechanisms, similar to those described in mammals. These include 1) the electrogenic sensor (González et al., 2009), evaluated by mRNA abundance of sodium-glucose linked transporter type 1 (SGLT-1); 2) the nuclear receptor sensor (Mitro et al., 2007) evaluated by mRNA abundance of liver X receptor (LXR); and, 3) the taste receptor sensor (Nakagawa et al., 2009), evaluated by mRNA abundance of gustducin (Gnat3). In a third series of experiments, we evaluated changes in the same parameters described in experiment 2 but in the presence of two well known inhibitors of glucose transport viz. cytochalasin B (GLUT inhibitor) and phlorizin (SGLT inhibitor). Finally, we carried out histochemical studies to substantiate and localize several of the main components of the glucosensing in head kidney tissue.

Materials and methods

Fish

Rainbow trout (Oncorhynchus mykiss Walbaum) were obtained from local fish farms either in the Netherlands (Forellenkwekerij De Keijzersberg, Blitterswijck, experiment 1) or Spain (A Estrada, experiments 2 and 3, and immunohistochemical studies). Fish were maintained for 15 days under laboratory conditions and a 12:12 L:D photoperiod in
dechlorinated tap water at 17 °C. Fish mass was 149 ± 4 g. Fish were fed once daily (10:00 h) to satiety with commercial dry fish pellets (Dibaq-Diproteg; 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 (2010/63/EU), and of the Governments of The Netherlands (code 2011139.a, Lelystad) and Spain (RD1201/2005) for the use of animals in research. Animal protocols were approved by the Animal Care Committees at Universities of Nijmegen and Vigo.

**Experimental design**

We used head kidney tissue (acknowledging the heterogeneity of the tissue) rather than isolated interrenal cells to avoid the possible dedifferentiation, which is usually associated with cell isolation. In addition to the convenience of collecting head kidneys as source of cortisol producing cells, the specificities of our assays and histology allowed us to define our findings unequivocally.

**Experiment 1. In vitro superfusion of head kidneys at different glucose concentrations in the absence/presence of ACTH**

Fish were fasted for 24h before treatment to ensure basal hormone levels were achieved. After anaesthesia with 2-phenoxyethanol (0.5% v/v), fish were sacrificed by decapitation, and head kidneys (left and right) were removed carefully and placed on a cheese-cloth filter in a superfusion chamber gassed with a 0.5% CO₂/99.5% O₂ mixture. Tissues (approx. 100 mg) were superfused with modified Hanks’ medium (92.56 mM NaCl; 3.63 mM KCl, 2.81 mM NaHCO₃, 0.85 mM CaCl₂, 0.55 mM MgSO₄, 0.4 mM KH₂PO₄, 0.23 mM Na₂HPO₄, 7.5 mM HEPES, 0.03% (w/v) bovine serum albumin, pH 7.15) at three different concentrations of D-glucose: 2, 4 and 8 mM (indicative of hypo-, normo- and hyperglycaemic conditions in rainbow trout), which was pumped through the chambers at 30 µl/min by a multichannel peristaltic pump (Watson-Marlow, Wilmington, MA, USA). All glucose treatments were evaluated in each replicate of head kidneys used. After 150 min, when cortisol release had reached a steady state, the medium was supplemented for 20 min with human ACTH (Sigma, St Louis, Mo, USA) at a concentration of 3.3x10⁻⁷M determined in a previous assay, in agreement with previous studies (Brodeur et al., 1998), except in control chambers where no ACTH was supplied. This procedure was repeated 8 times for each glucose concentration (2, 4, and 8 mM). Five-, ten- or thirty-minute fractions were
collected during 4 hours and stored at -80°C for cortisol assay. Area under curve (AUC) was calculated following the trapezoidal rule using the SigmaPlot software.

Experiment 2. *In vitro* incubation of head kidney tissue at different glucose concentrations in the absence/presence of ACTH

The superfusion experiments are not suitable to evaluate the mechanisms involved in the relationship between cortisol synthesis and release and glucose levels; static incubations were used to explore the underlying mechanisms.

Every morning of an experiment, fish that had been fasted for 24h (to ensure basal levels of metabolic hormones were achieved) were dip-netted, anaesthetized with MS-222 (50 mg·l−1) buffered to pH 7.4 with sodium bicarbonate, weighed and sacrificed by decapitation. Head kidneys were removed and rinsed by immersion in modified Hanks’ medium. In order to have enough mass, tissues were pooled from 3-4 fish. On each pool, tissues were finely sliced in a chilled Petri dish and mixed, and then placed in other Petri dishes containing 100 mL of modified Hanks’ medium.g⁻¹ tissue (gassed with a 0.5% CO₂/99.5% O₂ mixture) at three different concentrations of D-glucose: 2, 4 and 8 mM during 1.5 hours to ensure basal levels of cortisol release. After this time, tissue was placed in 48-well culture plates (25 mg of tissue in 250 µl of modified Hanks’ medium per well) at 2, 4 and 8 mM of glucose alone or in the presence of 3.3.x10⁻⁷M (3.3 x 10⁵ IU) ACTH (Sigma) to assess differences in response to glucose under constitutive (basal) and regulated (ACTH stimulated) conditions. All glucose treatments were assessed at the same time in each replicate of head kidneys used. After 10, 30 or 60 min incubation, tissues were quickly removed, snap-frozen in liquid nitrogen, and stored at -80°C until assayed whereas the medium was also taken for cortisol assessment. The sampling times used were selected based on the dynamics of cortisol release observed in experiment 1 after addition of ACTH.

For each experiment, 2 different sets of tissue pools (2 treatments x 3 glucose concentrations x 3 sampling times) were used. The first set was used to assess enzyme activities (GK, GSase, and PK) and metabolite levels (glucose and glycogen) whereas the second set was used for quantification of mRNA abundance. Cortisol levels in the medium were evaluated in all samples from the two sets used. The same procedure was performed in 4 independent replicates per set (N=4).

Experiment 3. *In vitro* incubation of head kidney tissue at 8 mM glucose with inhibitors of glucose transport in the presence of ACTH
Samples were obtained and processed as described in experiment 2. Tissue was placed in 48-well culture plates (25 mg of tissue in 250 µl of modified Hanks´medium per well) at 8 mM glucose alone (control) or in the presence of 3.3 x 10^{-7} M ACTH, or in the presence of 3.3 x 10^{-7} M ACTH and 10 µM cytochalasin B, or in the presence of 3.3 x 10^{-7} M ACTH and 1 mM phlorizin. The concentrations of inhibitors were selected based on previous studies on glucose transport in fish (Soengas and Moon, 1995, 1998). After 10, 30 or 60 min incubation, tissues were quickly removed, snap-frozen in liquid nitrogen, and stored at -80°C until assayed whereas the medium was also taken for cortisol assessment.

In each experiment, 2 different sets of tissue pools were used. The first set was used to assess enzyme activities (GK, GSase, and PK) and metabolite levels (glucose and glycogen) whereas the second set was used for quantification of mRNA abundance. Cortisol levels in the medium were evaluated in all samples from the two sets used. The same procedure was performed in 4 independent replicates per set (N=4).

Analytical procedures
Cortisol measurement
In experiment 1 cortisol was measured by radioimmunoassay. A 96-well micro-assay plate was incubated overnight at 4°C with 100 µl of cortisol antibody (Abcam, Cambridge, UK) diluted 1:2000 in coating buffer (50mM NaHCO_3, 50mM Na_2CO_3, and 0.02 % NaN_3). After washing with wash buffer (100mM Tris, 0.9% NaCl, and 0.02% NaN_3), the plate was incubated for 1 hour at 37°C with 100 µl of blocking buffer (normal calf serum at 0.25% in wash buffer). 10 µl of standards, plasma or superfusion fractions were incubated during 4 hours with ^3H-cortisol tracer (Perkin Elmer, Waltham, MA, USA) diluted in assay buffer (100mM Tris, 0.9% NaCl, 0.02% NaN_3, and 0.1% ANS, set pH to 7.4 with HCl). This RIA assay had been validated before for the measurement of cortisol in culture medium (Gorissen et al., 2012). In experiments 2 and 3 cortisol levels were measured by ELISA using a commercially available kit (Cayman, Ann Arbor, MI, USA).

Assessment of metabolite levels and enzyme activities
Samples used (approx. 20 mg) to assess metabolite levels were homogenized immediately by ultrasonic disruption in 7.5 vol of ice-cooled 6% perchloric acid, and neutralized (using 1 mol·l^{-1} potassium bicarbonate). The homogenate was centrifuged, and the supernatant used to assay tissue metabolites. Tissue glycogen levels were assessed using the method of Keppler and Decker (1974). Glucose obtained after glycogen breakdown (after
subtracting free glucose levels) was determined with a commercial kit (Biomérieux, Grenoble, France). Samples for enzyme activities (approx. 25 mg) 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). The homogenate was centrifuged and the supernatant used immediately for enzyme assays. Enzyme activities were determined using a microplate reader INFINITE 200 Pro (Tecan, Männedorf, Switzerland) 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 relative to protein content of the sample (activity per mg protein). Protein was assayed in triplicate in homogenates using microplates according to the bicinchoninic acid method with bovine serum albumin (Sigma) as standard. GK (EC 2.7.1.2), GSase (EC 2.4.1.11), and PK (EC 2.7.1.40) activities were determined as described previously (Polakof et al., 2007a,2008a,b,c). Enzyme activities were assessed at maximum rates by preliminary tests to determine optimal substrate concentrations.

mRNA abundance analysis by real-time quantitative RT-PCR

Total RNA was extracted from tissues (approx. 20 mg) using Trizol reagent (Life Technologies, Grand Island, NY, USA) and treated with RQ1-DNAse (Promega, Madison, Wi, USA). Two µg total RNA were reverse transcribed into cDNA using Superscript II reverse transcriptase (Life Technologies) and random hexaprimers (Life Technologies). Gene expression levels were determined by real-time quantitative RT-PCR (q-PCR) using the iCycler iQ™ (BIO-RAD). Analyses were performed on 1 µl cDNA using the MAXIMA SYBR® Green qPCR Mastermix (Fermentas, Vilnius, Lithuania), in a total PCR reaction volume of 15 µl, containing 50-500 nM of each primer. Abundance of GK, GLUT-2, Kir6.x-like, and SUR-like mRNAs was determined as previously described (Polakof et al., 2008a,c); mRNA abundance of 3βHSD, 11βH, LXR, P450sc, SGLT-1, and StAR was determined as previously described by other authors in the same species (Geslin and Auperin, 2004; Geurden et al., 2007; Cruz-García et al., 2009) and mRNA abundance of Gnat3 was determined using specific primers developed for rainbow trout based on available sequences (CU073912, Sigenae database, INRA; courtesy of Dr. S Polakof, INRA Clermont/Theix, France). Relative quantification of the target gene transcripts was done using elongation
factor 1α (EF1α) gene expression as reference, which was stably expressed in this experiment. Sequences of the forward and reverse primers used for each gene expression are shown in Table 1.

Thermal cycling was initiated with incubation at 95°C for 15 min using hot-start iTaq® DNA polymerase activation; 40 steps of PCR were performed, each one consisting of heating at 95°C for 15s for denaturing, and at specific annealing temperatures (Table 1) for 30s and extension at 72°C for 30s. Following the final PCR cycle, melting curves were systematically monitored (55°C temperature gradient at 0.5°C/s from 55 to 95°C) to ensure that only one fragment was amplified. Each sample was analyzed in triplicate. All the replicates of each sample were located in the same plate for each gene to allow comparisons. We included in all the plates the standard curve (by triplicate), and controls for NTC and RT negative control (by duplicate). Only efficiency values between 85-100% were accepted (the R² for all the genes assessed was always higher than 0.985). Relative quantification of the target gene transcript with the EF1α reference gene transcript was made following the Pfaffl method (2001).

**Immunohistochemistry**

Fish were anesthetized with MS-222, their head kidneys were excised, and small pieces were immersion-fixed in Bouin’s fluid or in 4% paraformaldehyde in 0.1M phosphate-buffered saline (PBS) at pH 7.4 for 24 h at 4°C. Later, pieces were paraffin-embedded and sections of 6-12 µm thick were stained using the hematoxylin-eosin (H-E) to study the overall structure. Other head kidney pieces were cryo-protected in 30% sucrose and were embedded in Tissue-Tec OCT compound (Sakura, Torrance, CA, USA). Horizontal and transversal sections (20 µm) were made on a cryostat. The primary antibodies used in this study were polyclonal rabbit anti-GK (Santa Cruz Biotechnology, Santa Cruz, CA, USA), polyclonal rabbit anti-SGLT-1 (Millipore, Billerica, MA, USA) and monoclonal mouse anti-TH (tyrosine hydroxylase, Millipore). The specificity of GK and SGLT-1 antibodies was previously tested by western blotting (Polakof et al., 2010).

Sections were processed as follows: 1) blocking endogenous peroxidase activity with 3% H₂O₂ for 30 min.; 2) pre-incubation with 0.1% bovine serum albumin (BSA) for 1 h, to inhibit non-specific reactivity; 3) incubation with anti-GK (1:100), anti-SGLT-1 (1:100) and anti-TH (1:200) antibodies overnight at room temperature in a humid chamber; 4) incubation with biotinylated goat anti-rabbit IgG (GAR 1:100, Vector, Burlingame, CA, USA) for GK and SGLT-1 and biotinylated goat anti-mouse IgG (GAM, Sigma, 1:100) for TH; 5) after
rinsing in PBS, the tissue was incubated with ABC-complex (Vector, 1:100); 6) the reaction was developed by incubation with 3,3′-diaminobenzidine (DAB, Sigma, 0.003%) and H₂O₂ (0.01%). All dilutions were made in PBS. Finally, some sections were counterstained with Mayer’s hematoxylin solution, dehydrated and cover-slipped with DEPEX mounting medium. Negative controls were run by omitting primary antisera during incubation of tissues.

To study co-localization of GK, SGLT-1 and TH, selected sections were processed for double immunofluorescence following the steps 1-2 as already described. Then, sections were incubated in a cocktail of the two antibodies SGLT-1/TH, GK/TH (dilution already described) and then with cocktail of secondary specific antibodies (Alexa Fluor 488 GAR-conjugated and Alexa Fluor 594 GAM-conjugated, Life Technologies) diluted 1:400 in PBS. After incubation, sections were washed in PBS and cover-slipped using Prolong Gold with DAPI (Life Technologies) to delay fluorescence fading. For co-localization of SGLT-1/GK, sections were incubated sequentially with anti-SGLT1, secondary antibody (Alexa Fluor 488 GAR-conjugated) observed and photographed. Then, the same sections were incubated with anti-GK followed by secondary specific antibody (Alexa Fluor 594 GAR-conjugated).

Slides were observed and photographed with an Olympus photomicroscope (BX51) equipped with digital camera (Olympus DP71). Confocal images were acquired with a spectral laser confocal microscope Leica SP5X.

**Statistics**

Comparisons among groups were carried out using three-way ANOVA with glucose concentration, presence/absence of ACTH, and time as main factors. Only in those cases where a significant effect was noted post-hoc comparisons were carried out by a Student-Newman-Keuls test, and differences were considered statistically significant at \( P < 0.05 \). When necessary data were log transformed to fulfill the conditions of the analysis of variance.

**Results**

**Experiment 1**

Significant time, ACTH, and glucose effects (\( P < 0.001 \)) were noted using a three-way ANOVA. The time course, and the area under the curve (AUC) of the time course of cortisol released from head kidney tissue displayed no major changes with the increase in glucose.
concentration in the medium when ACTH was not present; however, in the presence of ACTH cortisol release increased in parallel with the increase of glucose (Fig. 1).

**Experiment 2**

Cortisol released to the medium displayed significant time, ACTH, and glucose effects as well as significant interactions (Table 2). Levels were higher after 60 min of incubation at 2 mM glucose either in the absence or presence of ACTH, and after 30 min at 8 mM glucose in the presence of ACTH (Fig. 2); in the presence of ACTH higher cortisol levels were noted at 8 mM glucose after 30 min and at 2 mM glucose after 60 min (Fig. 2).

mRNA levels of genes related to cortisol synthesis (StAR, P450scc, 3\(\beta\)HSD, and 11\(\beta\)H) displayed glucose, ACTH, and time effects (Table 2). In the absence of ACTH the 4 parameters displayed at 8 mM glucose higher values at 10 min than at 30 or 60 min of incubation, and at 2 mM glucose lower values were noted after 60 min incubation for StAR and P450scc mRNA levels (Fig. 3). In the presence of ACTH higher values were noted for the 4 parameters after 10 min of incubation at 4 and 8 mM glucose (Fig. 3). Levels at 8 mM glucose were higher than those at 2 and 4 mM glucose after 10 min of incubation in the absence of ACTH whereas in the presence of ACTH a dose-dependent increase was noted in the four parameters assessed (Fig. 3).

Glucose levels displayed ACTH and glucose effects, and glycogen levels displayed glucose effects (Table 2). Glucose levels (Fig. 4A) were higher at 8 mM glucose than at 2 and 4 mM glucose in the absence of ACTH whereas a dose-dependent increase was noted in the presence of ACTH. Glycogen levels (Fig. 4B) were lower at 2 mM glucose than at 4 and 8 mM glucose after 10 min incubation in the absence of ACTH whereas levels were higher at 8 mM glucose than at 2 and 4 mM glucose after 60 min incubation in the presence of ACTH.

The activities of GK and GSase were affected by time and glucose and glucose x time interaction whereas PK activity displayed time effects (Table 2). GK activity (Fig. 5A) in the absence of ACTH increased after 60 min at 4 mM glucose compared with 30 min, and after 30 min at 8 mM glucose compared with 10 min whereas in the presence of ACTH activity was higher after 60 min at 2 and 8 mM glucose and lower after 30 min at 4 mM glucose; in the absence of ACTH the activity was lower at 2 mM glucose than at 4 mM glucose after 60 min, and also lower at 2 than at 4 mM glucose after 60 min whereas in the presence of ACTH activity was lower at 2 mM glucose than at 4 and 8 mM glucose after 10 and 60 min of incubation. GSase activity (Fig. 5B) increased with time at 2 mM glucose in the absence of ACTH whereas activity decreased with time at 8 mM glucose both in the absence or presence
of ACTH; the activity at 4 and 8 mM glucose was lower than that at 2 mM glucose after 30 and 60 min incubation both in the absence or presence of ACTH. PK activity (Fig. 5C) was higher after 30 min of incubation at 4 mM glucose (in the absence of ACTH) or 2 mM glucose (in the presence of ACTH).

mRNA levels of Kir6.x-like and SUR-like were affected by ACTH whereas mRNA levels of GK, GLUT-2, Kir6.x-like, and SUR-like were affected by time and glucose (except GLUT-2 for glucose) as displayed in Table 2. GK mRNA levels (Fig. 6A) were lower after 60 min of incubation at 8 mM glucose in the absence of ACTH whereas in the presence of ACTH levels were higher after 30 and 60 min at 4 mM glucose and after 30 min at 8 mM glucose; levels at 8 mM glucose were higher than those at 2 and 4 mM glucose after 15 and 30 min of incubation and lower after 60 min of incubation in the absence of ACTH and higher after 30 min of incubation in the presence of ACTH; moreover, levels were higher at 4 mM glucose than at 2 mM glucose after 10 min of incubation in the absence of ACTH and after 30 min of incubation in the presence of ACTH. GLUT-2 mRNA levels (Fig. 6B) increased after 30 and 60 min of incubation at 2 and 4 mM glucose both in the absence or presence of ACTH whereas at 8 mM glucose mRNA levels were higher after 30 min incubation in the absence of ACTH. Kir6.x-like mRNA levels (Fig. 6C) and SUR-like mRNA levels (Fig. 6D) increased after 30 and 60 min of incubation at 4 and 8 mM glucose in the absence of ACTH and at 2 and 4 mM glucose in the presence of ACTH; mRNA levels of both genes were higher at 2 mM glucose than at 4 and 8 mM glucose after 30 and 60 min of incubation in the presence of ACTH.

mRNA levels of LXR and Gnat-3 displayed no significant effects of glucose treatment though they were affected by time whereas SGLT-1 mRNA was affected by ACTH, time, and glucose (Table 2). SGLT-1 mRNA levels (Fig. 7A) increased after 60 min of incubation at 2 and 8 mM glucose in the absence of ACTH and at 2 and 4 mM glucose in the presence of ACTH; levels at 8 mM glucose were higher than those at 2 and 4 mM glucose in the absence of ACTH whereas levels at 2 mM glucose were higher than those at 4 and 8 mM glucose after 10 and 60 min of incubation in the presence of ACTH. LXR mRNA levels (Fig. 7B) were higher after 30 and 60 min of incubation at 4 mM glucose and after 30 min of incubation at 8 mM glucose in the absence of ACTH. Gnat3 mRNA levels (Fig. 7C) increased after 60 min of incubation at 2 and 4 mM glucose in the absence or presence of ACTH.

Experiment 3
The presence of phlorizin did not induce significant changes in any of the parameters assessed (data not shown). The effects of the presence of cytochalasin B on the parameters assessed in head kidney at 8 mM glucose are shown in table 3. Levels of cortisol and glycogen and mRNA abundance of P450scc, 11 βH and GLUT-2 that increased in the presence of ACTH returned to values similar to those of controls when cytochalasin B was present. GSase activity and GK mRNA abundance decreased in tissues treated with ACTH and cytochalasin B compared with controls and tissues treated with ACTH alone.

**Immunohistochemical studies**

In sections stained with haematoxylin-eosin, haematopoietic, interrenal, and chromaffin cells were easily recognizable (Fig. 8). In the areas surrounding vessels we observed interrenal (polygonal with rounded nuclei and dense cytoplasm and forming cords or follicles) and chromaffin (irregular or elongated in shape with light cytoplasm and are arranged in groups or cords) cells, which can be contiguous but are never mingled. GK immunoreactivity was present in interrenal cells but not in chromaffin cells. In contrast, SGLT-1 immunoreactivity was present in most (but not all) chromaffin cells.

In the colocalization studies (Fig. 9) we observed that TH co-localized with SGLT-1 in most cells but never co-localized with GK. In the cells where GK was present we observed a co-localization with SGLT-1.

**Discussion**

A well established paradigm in stress physiology is that corticosteroids stimulate glucose production to fuel metabolic processes for reestablishing homeostasis (Wendelaar Bonga, 1997). However, an allostatic control of cortisol levels requires adjustment of set points for cortisol production and release. Levels of circulating metabolites like glucose could be involved in such mechanism through either inhibitory or stimulatory effects. According to this model ACTH-stimulated cortisol release from head kidney of rainbow trout was clearly modulated by glucose concentration since increasing glucose levels elicited increased cortisol release (more clear in superfusion experiments) whereas ACTH-stimulated cortisol release was inhibited by the presence of cytochalasin B (an inhibitor of GLUT glucose carrier). These results apparently disagree with results obtained in zebrafish where cortisol levels were dependent on glucose in the absence of ACTH (Powers et al., 2010) though the study in
zebrafish evaluated changes in whole body content, not in medium levels as studied here and used much higher glucose concentration than that used in the present study. Since ACTH levels increase under stress conditions in fish (Wendelaar Bonga 1997) our results may indicate that the release of cortisol in response to increased glucose concentration occurs only under stress conditions. This situation would be comparable to that already reported in mammals where an additional stress is needed to increase cortisol release in response to an increase in glucose levels (Kirschbaum et al., 1997). However, in previous studies we observed that stress induced by high stocking density in rainbow trout did not result in a parallel response of cortisol levels in plasma to increased glucose levels (Conde-Sieira et al., 2010a,b), which could be related to the fact that cortisol levels in plasma are the net result of release and breakdown as well as clearance by excretion, and the balance determines plasma levels. Moreover, the response of cortisol levels to changes in glucose concentration could be dependent upon the type of stress and/or exposure to ACTH.

The increased release of cortisol from head kidney could be related to an enhanced production, and therefore we evaluated mRNA abundance of several genes involved in cortisol synthesis such as StAR, P450scc, 3βHSD, and 11βH whose changes in mRNA levels are known to mirror those of cortisol release in rainbow trout (Aluru and Vijayan 2006; Hagen et al., 2006). The presence of ACTH enhanced mRNA levels of P450scc, 11βH, and StAR under normoglycaemic conditions (4 mM glucose), which is in agreement with results previously obtained in rainbow trout (Geslin and Auperin, 2004; Aluru and Vijayan 2006). Furthermore, mRNA levels of genes related to cortisol synthesis increased in general in parallel with the increase of glucose concentration in the medium in the presence of ACTH thus supporting glucose modulation of ACTH-stimulated cortisol synthesis. In the absence of ACTH, only 8mM glucose activated these transcripts suggesting that basal cortisol can modulate transcript levels but only under hyperglycemic conditions, which is also in agreement with the increase noted in cortisol levels under the same condition. It is also interesting to mention that the modulation of glucose levels was clearly observed at short times of incubation, 10 min, since from that time onwards mRNA levels tended to be similar. This is probably suggesting a fast regulation of gene expression, which is later followed by changes in cortisol itself that showed higher levels thereafter (30 min). The effect of ACTH on genes involved in cortisol synthesis was also maximal in those short time periods. Further support to the relationship between glucose levels and cortisol synthesis comes from the results obtained in the presence of cytochalasin B that counteracted the increase induced by ACTH in the mRNA abundance of P450scc and 11βH.
Could glucosensors have a role in the connection between increased glucose levels and increased synthesis and release of cortisol? The best known glucosensor mechanism in mammals, the GK-mediated glucosensor (Marty et al., 2007), requires glucose uptake through the low-affinity glucose transporter GLUT-2, glucose phosphorylation by GK, and subsequent metabolism of glucose through glycolysis to increase the intracellular ATP/ADP ratio. This leads to the closure of $K_{ATP}$ channels, membrane depolarization, the entry of $Ca^{2+}$, which triggers increased neuronal activity and neurotransmitter secretion in brain regions and insulin release in pancreatic β-cells. We have characterized in hypothalamus, hindbrain, and Brockmann bodies (main accumulation of pancreatic endocrine tissue) of rainbow trout the presence of a similar GK-mediated glucosensor system (see review Polakof et al., 2011).

The possibility of a GK-mediated glucosensor system present in interrenal cells that could relate glucose detection to cortisol release is supported by our immunohistochemical studies in head kidney. In the areas surrounding blood vessels we observed two different cell types that can be identified as interrenal and chromaffin cells in agreement with previous studies in rainbow trout (Gallo and Civinini, 2001). In chromaffin cells (also identified by TH immunoreactivity) SGLT-1 was present in 60-70% of them, and GK was not detected. In interrenal cells GK and SGLT-1 were co-localized. The presence of GK in interrenal cells provides direct evidence for a relationship between cortisol biosynthesis and glucosensing through a glucosensor GK-related mechanism. Therefore, we assessed in head kidney slices several of the parameters related to GK-mediated glucosensing in response to increased glucose concentration. Changes observed in several of those parameters are in agreement with the functioning described in other glucosensing tissues in rainbow trout (Polakof et al., 2007a,b,2008a,b,c), such as the increase in glucose and glycogen levels, the increase of GK mRNA levels or the decrease in mRNA abundance of the components of the $K_{ATP}$ channel (Kir6.1-like and SUR-like) in response to increased glucose levels.

In contrast to those parameters, no changes were noted in other parameters such as GK activity or mRNA abundance of GLUT-2, that usually increase in response to raised glucose concentration in other glucosensing tissues in rainbow trout (Polakof et al., 2008a,b,c; Conde-Sieira et al., 2010a,b,2011). More surprising was the finding that GSase activity that normally increase in glucosensor tissues of mammals (Marty et al., 2007) and fish (Polakof et al., 2008a,b,c; Conde-Sieira et al., 2010a,2011) under increased glucose concentrations actually decreased in head kidney.

Further support to the presence of glucosensor mechanisms based on GK come from the results obtained when head kidney slices were incubated in the presence of a GLUT-2
antagonist like cytochalasin B. Cytochalasin B treatment in the presence of ACTH reversed the response of several parameters (glycogen levels and mRNA abundance of GK and GLUT-2) to ACTH though no changes were noted in others (even in the case of GSase activity an agonistic effect was noted). In general, it seems that several of the responses of parameters related to glucosensing are blocked by the inhibition of glucose transport through GLUT-2, one of the compulsory steps of glucosensing.

Therefore, not all of the components of a putative GK-mediated glucosensor system seem to be operational as predicted from a mammalian model in head kidney but several of them actually responded to increased glucose levels in a way similar to that previously addressed in other glucosensing tissues in rainbow trout. The lack of response in several parameters could be related to the fact that head kidney is a heterogeneous tissue composed of lymphoid cells, interrenal cells, melanomacrophages and chromaffin cells (Wendelaar Bonga 1997; Mommsen et al., 1999). In fact, Hontela et al (2008) described that only 0.01% of cells in rainbow trout head kidneys were interrenal cells. The fact that only few of the cells (interrenal) may possess glucosensing mechanisms related to GK would help to explain why not so clear changes were noted in several parameters since values obtained in this study are the result of the dilution effect of different cell types available in the tissue assessed. The differential expression patterns observed in the immunohistochemical studies would also support such contention. Moreover, GK is also present in other cell types in head kidney (such as in tubular cells of nephrons as observed in our immunohistochemical studies) whose response to glucose may be different than that occurring in interrenal cells.

We have no explanation regarding the connection between activation of a putative GK-mediated glucosensor mechanism in interrenal cells and increased cortisol release. In pancreatic endocrine tissue, the activation of glucosensor system based on GK induces the entry of calcium through L-type calcium channels resulting in subsequent insulin release. A comparable mechanism (though certainly operating through other effectors) may be present in interrenal cells of rainbow trout.

Other glucosensor systems described in mammalian brain regions and endocrine pancreas are the electrogenic sensor based on SGLT-1 (Gonzalez et al., 2009), the nuclear receptor sensor based on LXR (Mitro et al., 2007), and the taste receptor sensor based on gustducin (Nakagawa et al., 2009). In contrast to the GK-mediated sensor, there is no clear evidence in fish regarding the presence of any of those sensors yet, with only preliminary evidence describing SGLT-1 in rainbow trout intestine (Polakof et al., 2010). We have evaluated the response of several parameters related to those putative glucosensor systems in
head kidneys of rainbow trout maintained under different glucose concentrations. The nuclear receptor and the taste receptor sensors seem to be present but not to be operational under the conditions used in the present studies since no changes were noted for mRNA abundance. As for the electrogenic sensor, it could be operational in the absence of ACTH since increased mRNA abundance of SGLT-1 was noted in response to increased levels of glucose. Moreover, in our immunohistochemical studies we observed that SGLT-1 co-localized with GK in interrenal cells. However, comparing the response of SGLT-1 mRNA with that of cortisol levels and cortisol-related genes that mainly responded to glucose in the presence of ACTH, it seems that it is not operational as sensor involved in cortisol release since in the presence of ACTH SGLT-1 mRNA abundance decreased. These results are also supported by the lack of effect of the SGLT inhibitor phlorizin on cortisol release and mRNA levels of genes expressing proteins involved in cortisol metabolism. Therefore, a differential involvement of these sensing mechanisms in constitutive and regulated pathways could be proposed.

In summary, we demonstrate that ACTH-induced release of cortisol by trout head kidney is modulated by glucose levels in a way that increased release occurs under high glucose levels and decreased release occurs when glucose transport is inhibited by cytochalasin B. The release of cortisol can be associated with increased synthesis since enhanced mRNA abundance of genes related to cortisol synthesis was also noted under conditions of increased glucose. These results indicate that a glucosensor may exist in interrenal cells of rainbow trout. Accordingly, we obtained multiple evidence regarding the presence of a glucosensor mediated by GK in head kidney, presumably in interrenal cells, since immunoreactivity for GK was always present in interrenal cells but absent in chromaffin cells, and increased glucose levels resulted in changes in several parameters related to GK-mediated glucosensing similar to those already reported in glucosensor tissues of the same species. In contrast, no changes compatible with those of glucose levels and cortisol release/synthesis in the presence of ACTH were noted for any other putative glucosensor mechanisms based on LXR, SGLT-1 or Gnat3. However, due to the heterogeneity of the tissue used caution must be taken since specific studies were not carried out in isolated interrenal cells and, therefore, the effects observed may be indirect not reflecting a direct relationship between glucose levels and steroidogenic capacity. In this way, mammalian studies have shown that glucose can modulate chromaffin cells capacity for catecholamine synthesis (Piskuric et al., 2008).
The results combined are the first evidence for a mechanism in fish linking synthesis and release of a non-pancreatic hormone like cortisol with circulating glucose levels, in a way comparable to that occurring in pancreatic β-cells responding to increased glucose levels with raised insulin release. The relationship was evident for the regulated (ACTH-dependent) pathway and this suggests that under acute stress conditions glucose is important for the regulation of cortisol synthesis and release. A coupling of ACTH-stimulated cortisol release to circulating glucose seems logical as a hyperglycemic state supports the cortisol surges (and their impact on glucose utilization) under acute stress conditions.

Acknowledgements

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References


Table 1. Nucleotide sequences of the PCR primers used to evaluate mRNA abundance by RT-PCR (qPCR)

<table>
<thead>
<tr>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>Annealing temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EF1α</td>
<td>GGG CAA GGG CTC TTTCAA GT</td>
<td>CGC AAT CAG CCT GAG AGGT</td>
</tr>
<tr>
<td>GK</td>
<td>GCACGGCTGAGATGCTCTTTG</td>
<td>GCCTTAACCCCTTGGTCAAG</td>
</tr>
<tr>
<td>GLUT-2</td>
<td>GTGGAGAAGGAGGCGCAAGT</td>
<td>GGCAACGGACACCATTGTAAGA</td>
</tr>
<tr>
<td>Gnat3</td>
<td>GCAAGACGTGCTGGAGACCA</td>
<td>ATGCGGGTGACTCCATTCAA</td>
</tr>
<tr>
<td>3βHSD</td>
<td>TCAAGGGGTTCAACCGTTCAAG</td>
<td>CCTCCCTTTTGGTCTTTGCTG</td>
</tr>
<tr>
<td>11βH</td>
<td>ATTTGCGCTGTACAGATTTGG</td>
<td>GGATGATGATGTCTTCTGACTG</td>
</tr>
<tr>
<td>Kir6.x-like</td>
<td>TGGGCTCTCTTCGCACTGT</td>
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<td>LXR</td>
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</tr>
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<td>CAGCGGTATCATCTTCAGCA</td>
</tr>
<tr>
<td>SGLT-1</td>
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<td>CTCATAACCTCCACCTCATTT</td>
</tr>
<tr>
<td>StAR</td>
<td>CTCTACAGCATATGAGGAAC</td>
<td>GCCTACCTCCCTGGCTTC</td>
</tr>
<tr>
<td>SUR-like</td>
<td>CGAGGACTGGCCGAGCA</td>
<td>GACCTTCACCTTGCATGCT</td>
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EF1α, elongation factor 1α; GK, glucokinase; GLUT-2, glucose facilitative transporter type 2; Gnat3, gustducin, 3βHSD, 3β-hydroxysteroid dehydrogenase; 11βH, 11β-hydroxylase; Kir6.x-like, inward rectifier K⁺ channel pore type 6.-like; LXR, liver X receptor; P450scn, cytochrome P450 cholesterol side chain cleavage; SGLT-1, sodium-glucose linked transporter type 1; StAR, steroidogenic acute regulatory protein; SUR-like, sulfonylurea receptor-like
Table 2. P-values obtained after three-way analysis of variance of parameters assessed in head kidneys of rainbow trout in experiment 3. Time (10, 30, and 60 min), ACTH (presence and absence), and glucose concentration (2, 4, and 8 mM) were the main factors. Glucose x time, Glucose x ACTH, and Time x ACTH are the first order interactions. Glucose x ACTH x Time is the second order interaction. All values are significantly different unless noted by a dash.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Time</th>
<th>ACTH</th>
<th>Glucose</th>
<th>Glucose x Time</th>
<th>Glucose x ACTH</th>
<th>Time x ACTH</th>
<th>Glucose x ACTH x Time</th>
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<td></td>
<td></td>
<td></td>
<td></td>
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<td>0.045</td>
<td>&lt;0.001</td>
<td>-</td>
<td>0.051</td>
<td>0.037</td>
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<td>-</td>
<td>0.017</td>
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<td>0.047</td>
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<td>-</td>
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<td>0.021</td>
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<td>0.033</td>
<td>0.037</td>
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<td>11βH mRNA</td>
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<td>0.042</td>
<td>0.022</td>
<td>-</td>
<td>0.045</td>
<td>&lt;0.001</td>
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<td>Glucose</td>
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<td>-</td>
<td>0.043</td>
<td>-</td>
<td>-</td>
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<td>-</td>
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<tr>
<td>GK activity</td>
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<td>0.014</td>
<td>0.001</td>
<td>-</td>
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<tr>
<td>GSase activity</td>
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<td>&lt;0.001</td>
<td>0.012</td>
<td>-</td>
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<tr>
<td>PK activity</td>
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<td>-</td>
<td>-</td>
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<td>GK mRNA</td>
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<td>0.033</td>
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<td>GLUT-2 mRNA</td>
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<td>Kir6.x-like mRNA</td>
<td>&lt;0.001</td>
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<td>SUR-like mRNA</td>
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<td>SGLT-1 mRNA</td>
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<td>Gnat-3 mRNA</td>
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Table 3. Average values of cortisol levels released to the incubation medium, parameters related to cortisol metabolism, and parameters related to glucosensing mediated by GK in head kidneys of rainbow trout incubated 10, 30 and 60 min at 15 °C in modified Hanks’ medium containing 8 mM D-glucose alone (Control) or in the presence of 3.3.x10^{-7}M ACTH or in the presence of 3.3.x10^{-7}M ACTH and 10 µM cytochalasin B. Results are shown as % of control values (control = 100%). Each value is the mean ± S.E.M. of 8 (cortisol) or 4 (remaining parameters) independent experiments carried out with pools of head kidneys from 3-4 different fish. Different letters indicate significant differences (P<0.05) among treatments.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>ACTH</th>
<th>ACTH + cytochalasin B</th>
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<tr>
<td>Cortisol levels</td>
<td>100.0 ± 8.89 a</td>
<td>180.3 ± 13.9 b</td>
<td>137.5 ± 7.73 a</td>
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<tr>
<td>Cortisol metabolism</td>
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<td></td>
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<tr>
<td>StAR mRNA</td>
<td>100.0 ± 13.9</td>
<td>101.5± 15.2</td>
<td>96.5± 12.9</td>
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<tr>
<td>P450scc mRNA</td>
<td>100.0 ± 6.56 a</td>
<td>181.1 ± 14.1 b</td>
<td>96.7 ± 8.60 a</td>
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<td>3βHSD mRNA</td>
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<td>114.7 ± 9.7</td>
<td>83.3 ± 10.6</td>
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<td>11βH mRNA</td>
<td>100.0 ± 9.78 a</td>
<td>167.5 ± 7.78 b</td>
<td>89.7 ± 15.01 a</td>
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<td>Glucosensing</td>
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<td>Glucose levels</td>
<td>100.0 ± 3.36</td>
<td>107.1 ± 3.25</td>
<td>114.4 ± 3.19</td>
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<td>Glycogen levels</td>
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<td>142.1 ± 10.2 b</td>
<td>81.7 ± 5.55 a</td>
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<td>78.5 ± 8.95</td>
<td>102.6 ± 5.99</td>
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<tr>
<td>GSase activity</td>
<td>100.0 ± 8.91 a</td>
<td>99.4 ± 10.4 a</td>
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<td>PK activity</td>
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<td>GLUT-2 mRNA</td>
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<td>SUR-like mRNA</td>
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<td>77.9 ± 8.48</td>
<td>146.7 ± 11.4</td>
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Figure legends

**Fig. 1.** Time course of cortisol levels (A) and area under curve (AUC) of the time course of cortisol levels (B) released to the incubation medium by head kidneys of rainbow trout superfused at 15 °C in modified Hanks’ medium containing 2, 4 or 8 mM D-glucose alone or in the presence for 20 min (after 150 min preincubation) of 3.3.x10^{-7} M ACTH. Each value is the mean ± S.E.M. of 8 independent experiments. % basal levels are the average cortisol levels measured in the incubation media once the superfusion became stabilized (in the interval time between 120 and 150 min). In B panel: different letters indicate significant differences (P<0.05) among glucose concentration within each treatment; *, Significantly different from group without ACTH at the same glucose concentration.

**Fig. 2.** Time course of cortisol levels released to the incubation medium by head kidney slices of rainbow trout incubated at 15 °C in modified Hanks’ medium containing 2, 4 or 8 mM D-glucose alone (No ACTH) or in the presence of 3.3.x10^{-7} M ACTH. Each value is the mean ± S.E.M. of 4 independent experiments. Comparisons among groups were carried out using three-way ANOVA with glucose concentration, presence/absence of ACTH, and time as main factors. Only in those cases where a significant effect was noted post-hoc comparisons were carried out by a Student-Newman-Keuls test. *, Significantly different from group incubated without ACTH at the same time and glucose concentration. Different letters indicate significant differences (P<0.05) among sampling times within each glucose concentration. #, Significantly different from group incubated with 2 mM glucose at the same sampling time (P<0.05); $, significantly different from group incubated with 4 mM glucose at the same sampling time (P<0.05).

**Fig. 3.** Time course of mRNA abundance of StAR (A), P450scc (B), 3βHSD (C), and 11βH (D) in head kidney slices of rainbow trout incubated at 15 °C in modified Hanks’ medium containing 2, 4 or 8 mM D-glucose alone (No ACTH) or in the presence of 3.3.x10^{-7} M ACTH. Each value is the mean ± S.E.M. of 4 independent experiments. Differences in mRNA levels between treatments are presented as an x-fold-change with respect to 2 mM glucose control (no ACTH) at 10 min. Expression results were normalised to EF1α mRNA levels (mRNA levels-no variation). Further details as in legend of Fig. 2.

**Fig. 4.** Time course of levels of glucose (A), and glycogen (B) in head kidney slices of rainbow trout incubated at 15 °C in modified Hanks’ medium containing 2, 4 or 8 mM D-glucose alone (No ACTH) or in the presence of 3.3.x10^{-7} M ACTH. Each value is the mean ± S.E.M. of 4 independent experiments. Further details as in legend of Fig. 2.

**Fig. 5.** Time course of the activities of GK (A), GSase (B), and PK (C) in head kidney slices of rainbow trout incubated at 15 °C in modified Hanks’ medium containing 2, 4 or 8 mM D-glucose alone (No ACTH) or in the presence of 3.3.x10^{-7} M ACTH. Each value is the mean ± S.E.M. of 4 independent experiments. Further details as in legend of Fig. 2.

**Fig. 6.** Time course of mRNA abundance of GK (A), GLUT-2 (B), Kir6.x-like (C), and SUR-like (D) in head kidney slices of rainbow trout incubated at 15 °C in modified Hanks’ medium containing 2, 4 or 8 mM D-glucose alone (No ACTH) or in the presence of 3.3.x10^{-7} M ACTH. Each value is the mean ± S.E.M. of 4 independent experiments. Differences in mRNA levels between treatments are presented as an x-fold-change with respect to 2 mM glucose control (no ACTH) at 10 min. Expression results were normalised to EF1α mRNA levels (mRNA levels-no variation). Further details as in legend of Fig. 2.
**Fig. 7.** Time course of mRNA abundance of SGLT-1 (A), LXR (B), and Gnat3 (C) in head kidney slices of rainbow trout incubated at 15 °C in modified Hanks’ medium containing 2, 4 or 8 mM D-glucose alone (No ACTH) or in the presence of 3.3 x 10^{-7} M ACTH. Each value is the mean ± S.E.M. of 4 independent experiments. Differences in mRNA levels between treatments are presented as an x-fold-change with respect to 2 mM glucose control (no ACTH) at 10 min. Expression results were normalised to EF1α mRNA levels (mRNA levels-no variation). Further details as in legend of Fig. 2.

**Fig. 8.** Histological sections of rainbow trout head kidney stained with H-E. The interrenal tissue is easily recognizable to be arranged in groups or cords (A, B). The interrenal cells are positive for GK (C, D) and SGLT-1 (E). The GK-ir lines the wall of the blood vessel (E). The chromaffin cells are identified by the positive reaction for TH (F). Dispersed in the head kidney there are small ganglia and nerve fibers also TH-immunoreactives (TH-ir) (results not shown). CC: chromaffin cells. IE: interrenal tissue. M: melano-macrophages. RT: renal tissue. V: blood vessel. Scale bars: 100 μm (A); 25 μm (B); 50 μm (C-F)

**Fig. 9.** Confocal photomicrographs of selected double labeled sections of rainbow trout head kidney showing the distribution/colocalization of SGLT1/TH (A-C), GK/TH (D-F) and SGLT-1/GK (G-I) immunoreactivity. Cells showing colocalization SGLT-1/TH and SGLT-1/GK appear in yellow. Note in green the intense GK-ir cells in the renal tubule; these cells are also SGLT-1-ir (results not shown). Scale bars: 25 μm
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