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Glucose homeostasis is impaired by a paradoxical interaction between metformin and insulin in carnivorous rainbow trout

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Polakof S, Skiba-Cassy S, Panserat S. Glucose homeostasis is impaired by a paradoxical interaction between metformin and insulin in carnivorous rainbow trout. *Am J Physiol Regul Integr Comp Physiol* 297: R1769–R1776, 2009. First published September 30, 2009; doi:10.1152/ajpregu.00369.2009.—Utilizing rainbow trout (*Oncorhynchus mykiss*) as a known model of a "glucose-intolerant" and poor dietary glucose user, we assessed glucose utilization in fish chronically receiving two molecules able to improve glucose homeostasis: insulin and metformin. Our objectives were to assess the ability of rainbow trout to deal with a glucose load and to improve glucose utilization in fish receiving a chronic administration of insulin plus metformin treatments. Fish received (implanted miniosmotic pumps) saline, insulin, metformin, and insulin plus metformin solution for 4 days and then were subjected to a glucose challenge (intraperitoneal injection) to study glucose homeostasis, analyzing plasma glycemia, mRNA levels of glucose metabolism-related proteins, insulin signaling, and glycogen levels in liver and muscle. Control fish received a saline pump implantation and saline intraperitoneal injection. We found no evidence that the "glucose intolerance" in this species could be linked to any of the molecular markers of metabolism in the tissues analyzed. By contrast, very interestingly, we show for the first time, that metformin is not only unable to improve glucose homeostasis in trout, but, in fact, its counteracts the effects of insulin, creating an "insulin resistance," especially in the muscle. These results make trout an attractive original model to study both insulin and metformin effect on biological systems.

fish; insulin; glucose metabolism; liver; muscle; metformin

CARNIVOROUS FISH SPECIES LIKE rainbow trout (*Oncorhynchus mykiss*) has been traditionally considered as "glucose intolerant" (21, 40), mainly because of the prolonged hyperglycemia experienced after a glucose load or intake of carbohydrate meals (2, 25). Despite its carnivorous feeding habits, with diets mainly composed of lipids and proteins, and with glucose intakes below 1% in the natural environment, a glucosensor system has been shown to exist in rainbow trout (32). In addition, trout fed carbohydrate-rich feeds show an efficient adaptation of glucose transport and utilization (oxidation and storage) in liver and muscle (4, 22, 26, 28). However, gluconeogenesis in the liver (27, 28) and glucose phosphorylation in the muscle (17) does not seem to be controlled by glucose as in mammals (6, 19, 35); in fish fed high levels of carbohydrates, the impaired postprandial downregulation of gluconeogenic enzymes resemble the insulin resistance observed in human patients with type II diabetes.

In this sense, although glucose is not the most potent insulin secretagogue in fish, hyperglycemia often leads to hyperinsulinemia (21). However, the mechanism by which insulin regulates plasma glucose levels in fish remains unknown, and the relative contribution of the main sensitive peripheral tissues to this hormone remains still to be clarified (23).

Metformin is an antidiabetic drug used extensively for the treatment of human type 2 diabetes, improving glucose homeostasis by its action on insulin sensitivity in the liver (mainly) and in the muscle. Although the inhibitory effects on hepatic glucose production is considered preponderant, it is likely that it is the interaction among the effect on the two different tissues that bring about the overall beneficial effect of metformin (8). Treatment with metformin was previously reported in two cyprinid species, the common carp and the zebrafish, both known to utilize glucose efficiently (7, 11). Also, in rainbow trout fed with a high-carbohydrate diet plus metformin, despite the improved glucose homeostasis, unexpected effects, such as the induction of gluconeogenic and lipogenic hepatic gene expression, were found (29).

In the present study, utilizing rainbow trout as model of glucose-intolerant fish, we assessed glucose utilization in fish chronically treated with molecules known to improve glucose homeostasis, such as insulin and metformin. Our objectives were then *1*) to assess at the molecular level the ability of rainbow trout to deal with a glucose load; *2*) to study the capacity to utilize glucose after chronic treatment with insulin or metformin; and *3*) to improve glucose utilization in trout receiving a chronic insulin plus metformin treatment. Rainbow trout were treated with saline, insulin, metformin, and insulin plus metformin solution for 4 days and then subjected to a glucose challenge to study glucose homeostasis, analyzing plasma glycemia, mRNA levels of glucose metabolism-related proteins (lipogenesis, gluconeogenesis, glycolysis, and glucose transport), insulin signaling, and glycogen levels in liver and muscle.

MATERIALS AND METHODS

Fish. Rainbow trout (*Oncorhynchus mykiss* Walbaum) were obtained from the French National Institute for Agricultural Research experimental fish farm facilities of Donzacq (Landes, France). Fish were maintained in tanks kept in open circuits with 17°C well-aerated water with a controlled photoperiod (12:12-h light-dark cycle) and were fed a commercial diet (T-3P classic, Trouw, France). Fish weight was 170 ± 4 g. The experiments were conducted following the Guidelines of the National Legislation on Animal Care of the French Ministry of Research (Decret no.

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2001-464 of May 29, 2001) and was approved by the Ethics Committee of INRA (according to INRA Decret no. 2002-36 of April 14, 2002).

Experimental protocols. For the chronic administration, fish were food deprived for 48 h and then implanted with 1007D Alzet miniosmotic pumps (Alza, Mountain View, CA, USA) containing either saline (control, $n = 16$), metformin (Sigma, $n = 16$), bovine insulin (Sigma, $n = 16$), or a metformin plus insulin solution ($n = 16$). To avoid solubility problems between insulin and metformin in the same pump, fish receiving both molecules were implanted with two pumps (one for insulin, one for metformin), while the rest of the groups were implanted also with a pump diffusing only saline solution. Mini-pump flow rate was established to be 0.39 μl/h, which at 17°C, should provide sustained release of 20 $mg \cdot kg^{-1} \cdot day^{-1}$ of metformin and 0.35 insulin $IU \cdot kg^{-1} \cdot day^{-1}$ for 11 days. Fish were first anesthetized, and their body weight was determined. Minipumps were inserted into the peritoneal cavity through a 1.0-cm incision made in the ventral midline at ~2.0 cm rostral of the pelvic fins. The incision was closed with one stitch, and an antibiotic gel was applied topically to the incision area. After 4 days, eight fish per group were intraperitoneally injected with either saline or glucose solution (250 mg/kg, Sigma), and 6 h after the injection, fish were randomly sampled as describer earlier (32). Blood was removed from the caudal vein into ammonium-heparinized syringes, centrifuged, and the recovered plasma was immediately frozen and kept at −20°C pending analyses. Liver and a sample of dorso-anterior white muscle were dissected and immediately frozen in liquid nitrogen and kept at −80°C pending analyses.

Molecular and biochemical analysis. Plasma glucose and triglyceride levels were determined using a commercial kit (Biomérieux, Marcy l'Etoile, France) adapted to a microplate format. Bovine insulin levels were measured using a commercial ELISA kit (Mercodia, Uppsala, Sweden). Because exogenous bovine and not endogenous trout insulin (expected to be extremely low in 6 day-fasted fish, see Ref. 18) levels were determined, we cannot discard possible mixed effects between both types of insulin in the present study. Tissue glycogen levels were determined following the method of Keppler and Decker (16). Plasma metformin was extracted as Amini et al. (1) and assessed spectrophotometrically using the method of Hassan et al. (9).

mRNA levels for proteins involved in glucose transport and metabolism were determined by real-time quantitative RT-PCR (q-PCR) (5, 18, 31), including GLUT1, GLUT2, GLUT4 (glucose facilitative transporters), glucokinase (GK), hexokinase (HK), pyruvate kinase (PK), 6-phophofructo-1-kinase (6PF1K), glucose 6-phosphatase (G6Pase), fructose 1,6-bisphosphatase (FBPase), phosphoenolpyruvate carboxykinase (PEPCK), fatty acid synthase (FAS), and glucose 6-phosphate dehydrogenase (G6PDH). Primers were designed to overlap an intron if possible (Primer3 software) using known sequences in trout nucleotide databases (GenBank and INRA-Sigenae), as previously described (29), except for 6PF1K in the liver (forward primer: GGTGGAGATGCACAAGGAAT; reverse primer: CTTGATGTTGTCCCCTCCAT; tcbk0069c.k.05_s.1) and muscle (forward primer: GGGACCTCGAGATGAACGTA; reverse primer: GAGGGCGAAAGATGAAGTCTG; tcad0007a.e.10_3.1.2.1). Quantification of the target gene transcript was done using *ef1α* gene expression as a reference (24), which was found to be stably expressed in this study. Relative quantification of the target gene transcript with the *ef1α* reference gene transcript was made following the Pfaffl method (30).

Protein extraction (20 μg of protein for liver and muscle) and Western blot analysis were developed (31) using anti-phospho-Akt Ser473 and anti-Akt antibodies (Cell Signaling Technology, Saint Quentin Yvelines, France), which have been shown to successfully cross-react with rainbow trout Akt protein (36).

Statistical analysis. Results are expressed as means ± SE ($n = 8$). For biochemical parameters, results were analyzed by two-way ANOVA (SigmaStat; SPSS, Chicago, IL) with injected treatments (saline or glucose) and implanted treatments (saline, insulin, metformin, or insulin plus metformin) as independent variables. For gene expression, data were analyzed by one-way ANOVA. When necessary, data were log-transformed to fulfill the conditions of the ANOVA. Post hoc comparisons were made using a Student-Newman-Keuls test, and differences were considered statistically significant at $P < 0.05$.

RESULTS

Plasma glucose levels are shown in Fig. 1*A*. After 4 days of infusion, fish implanted with insulin and insulin plus metformin and injected with saline solution displayed a significant hypoglycemia (2–2.5 mM), while fish implanted only with metformin were normoglycemic (~4 mM). Fish injected with glucose were all hyperglycemic, although plasma glucose levels were still lower (~1.8-fold) in the insulin-implanted fish compared with the other groups. A similar profile was found in liver glycogen levels (Fig. 1*C*), since glucose injection promoted glycogen storage in all of the groups, although the accumulation was lower (~2-fold) in the insulin-treated trout (for saline or glucose injection) than in the other groups. In saline-injected fish, glycogen storage in muscle (Fig. 1*D*) was promoted by insulin, metformin, or insulin plus metformin infusion during 4 days. However, when fish received glucose injection, glycogen storage increased nine-fold in saline-implanted trout, in contrast to the other pump treatments, in which glycogen stores diminished seven-fold with respect to the saline-implanted/glucose-injected group, but also with respect to their controls (saline-injected fish). No saline-implanted/glucose-injected changes were observed in plasma triglyceride levels (Fig. 1*B*) either due to the pump treatment or due to glucose/saline injection.

Molecular effects of glucose injection in saline, metformin, insulin, and metformin plus insulin-implanted trout. The effect of glucose injection on mRNA levels for proteins involved in glucose metabolism in implanted fish is shown in Table 1. In sham-implanted fish, glucose injection resulted in increased mRNA levels for proteins involved in glucose transport (GLUT2), glycolysis (HK, GK-1000-fold induction-, PK-L, 6PF1K-L), gluconeogenesis (PEPCK), and lipogenesis (FAS, G6PDH) in liver. However, in the same conditions, G6Pase transcript levels were downregulated. In muscle, no changes were noticed after glucose administration. In contrast, when insulin was infused, glucose injection promoted the opposite changes in liver. Then, these genes upregulated in the sham-infused group were downregulated (GLUT2, HK, PK-L, G6PDH) or their induction reduced (GK, 265-fold induction). For other genes (6PF1K-L, PEPCK, FAS), the induction promoted by glucose in the sham-infused context disappeared, either being unaffected (or with a slight reduction). Fish receiving metformin infusion for 4 days and then injected with glucose displayed few changes in mRNA levels, including GK induction (although less than in saline-infused fish), as well as 6PF1K-L and G6Pase downregulation. The results obtained in insulin plus metformin-implanted fish were globally intermediate between those obtained with insulin or metformin alone. Furthermore, we did not find changes in hepatic mRNA levels of proteins such as GLUT2, HK, 6PF1K-L, FBPase, and FAS, while GK (1550-fold induction) mRNA levels were always induced and G6Pase repressed. Other genes showed results more similar to the saline group (induced PK-L), while others were completely opposite to that group (repressed G6PDH and PEPCK). In the muscle, glucose transport in fish treated with

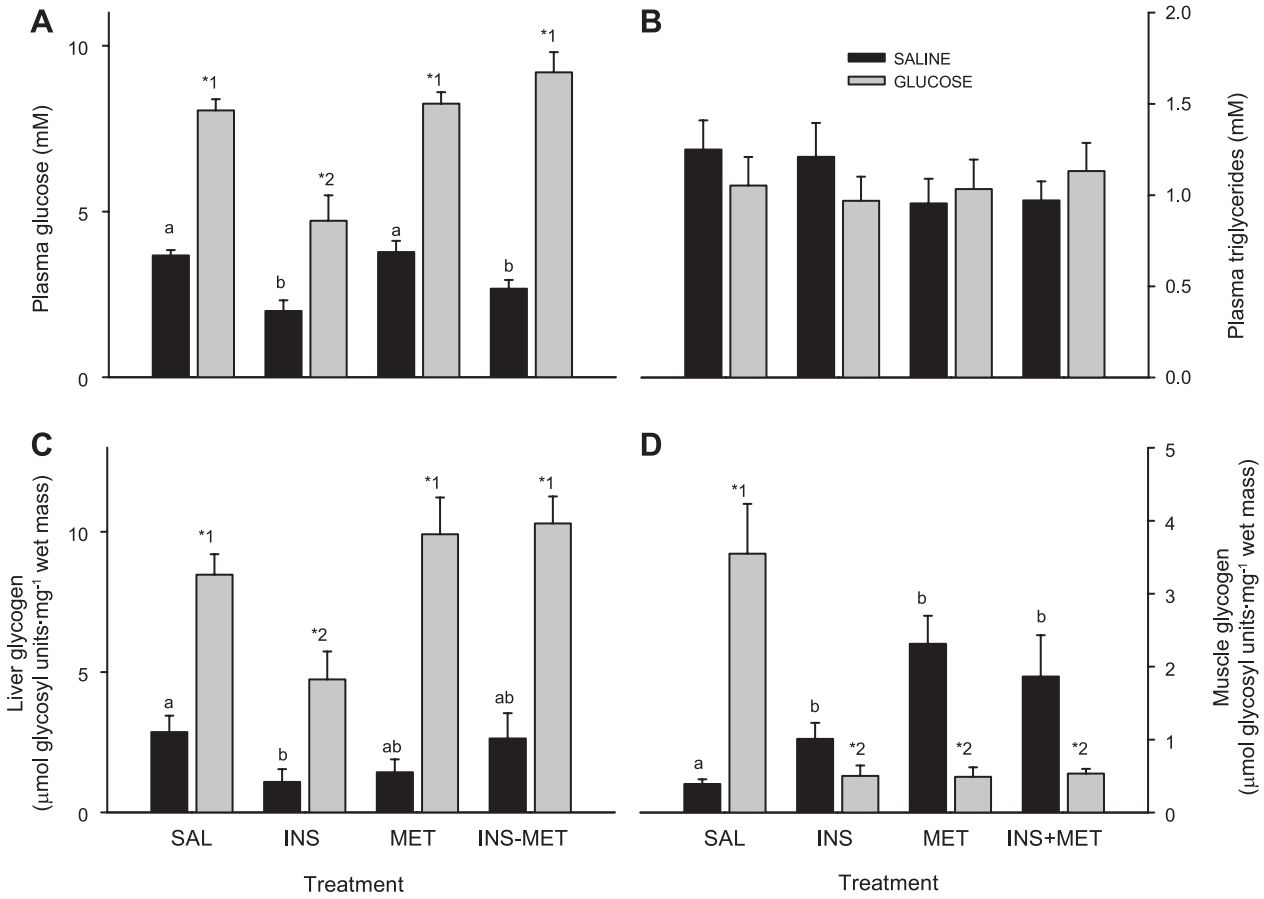

Fig. 1. Plasma glucose (*A*), plasma triglycerides (*B*), liver glycogen (*C*), and muscle glycogen (*D*) levels 6 h after intraperitoneal administration of saline or glucose solution (250 mg/kg) in fasted rainbow trout implanted with saline, insulin (1.4 IU/kg), metformin (80 mg/kg), or insulin plus metformin solution for 4 days. Results are expressed as means ± SE ($n = 8$) and were analyzed by two-way ANOVA followed by Student-Newman-Keuls multiple-comparison test. *Significant difference between saline- and glucose-injected fish for each implanted group ($P < 0.05$). Different letters indicate significant differences among implanted groups within the saline-injected treatment ($P < 0.05$). Different numbers indicate significant differences among implanted groups within the glucose-injected treatment ($P < 0.05$).

insulin plus metformin clearly maintained the profile observed in the insulin-infused group (increased mRNA levels for GLUTs), while other actors of glucose metabolism remained unaffected, as in the metformin-treated group.

Comparison of mRNA levels in fish receiving saline or glucose injection. Comparisons of mRNA levels for glucose metabolism-related enzymes in the liver of trout implanted with saline, insulin, metformin, or insulin plus metformin solution and injected with glucose are shown in Fig. 2. Overall, we found that after glucose injection, insulin seems to exert a minor action in hepatic transcript levels compared with the saline-injected group, since only GK and PEPCK were affected (down-regulated). In contrast, the effect of metformin after glucose administration was remarkable, repressing by 2-fold most of the hepatic genes related with glucose metabolism, including GK, 6PF1K-L, FAS, G6PDH, G6Pase, FBPase, and PEPCK. In contrast, other genes such as PK-L or GLUT2 were upregulated by metformin. The glucose administration in the context of combined treatment with insulin plus metformin seems to affect more the lipogenic and gluconeogenic pathways in the liver, since mRNA levels of all of the enzymes of these pathways were diminished as in the metformin-alone treatment. In contrast, mRNA levels of protein involved in glucose transport and glycolysis (except GK transcript levels which was upregulated) were unaffected by the combination of both in the hyperglycemic context.

Comparisons of mRNA levels of glucose metabolism-related enzymes in the muscle of trout implanted with saline, insulin, metformin, or insulin plus metformin solution and injected with glucose are shown in Fig. 3. We found that transcript levels of GLUTs in the muscle were increased in the insulin-treated fish, while lack of changes (GLUT4) or down-regulation (GLUT1) was observed in fish receiving metformin or insulin plus metformin. Glycolytic PF1K-M mRNA levels were down-regulated, inhibited by metformin alone and in the presence of insulin, as for PK-M (only diminished by the insulin plus metformin treatment).

In general, no differences were noticed between implanted fish when they were intraperitoneally injected with the saline solution, except for those infused with insulin, in which the expected effects of insulin were observed, and were not altered by metformin.

Focus on metformin-insulin interaction. Akt phosphorylation status in the liver and muscle of fish injected with glucose are shown in Fig. 4. In the liver, Akt phosphorylation increased in fish infused with insulin by twofold compared with the sham

Table 1. *Effects of intraperitoneal administration of glucose (6 h) in fish implanted with saline, insulin, metformin, or insulin plus metformin (4 days) on the level of expression of mRNA encoding hepatic and muscle genes*

	Saline	Insulin	Metformin	Insulin + Metformin
		Liver		
GLUT2	+1.4*	−1.5*	+1.0	+1.1
GK	+1027*	+265*	+534*	+1550*
HK	+1.6*	−1.6*	+1.0	+1.0
6PF1K-L	+2.1*	+1.3	−1.9*	+1.1
PK-L	+2.4*	−1.4*	+1.2	+1.5*
G6Pase	−4.1*	−7.5*	−3.8*	−7.2*
FBPase	+1.2	−1.2	−1.2	−1.4
PEPCK	+1.6*	−1.1	−1.2	−1.8*
FAS	+2.1*	−1.1	+1.2	−1.2
G6PDH	+1.8*	−1.5*	−1.1	−2.0*
		Muscle		
GLUT1	+1.1	+1.9*	+1.3	+1.2*
GLUT4	+1.0	+2.0*	+1.1	+2.5*
HK	+1.2	+1.6*	+1.1	+1.2
6PF1K-M	+1.3	+2.1*	−1.3	+1.0
PK-M	−1.2	+1.4*	+1.2	+1.1

Results are expressed as fold variation of the saline-injected treated group and were analyzed by one-way ANOVA followed by Student-Newman-Keuls comparison test. *Significant difference ($P < 0.05$). GLUT, glucose facilitative transporter; GK, glucokinase; HK, hexokinase; PK, pyruvate kinase; 6PF1K, 6-phophofructo-1-kinase; G6Pase, glucose 6-phosphatase; FBPase, fructose 1,6-bisphosphatase; PEPCK, phosphoenolpyruvate carboxykinase; FAS, fatty acid synthase; and G6PDH, glucose 6-phosphate dehydrogenase.

group. In contrast, Akt phosphorylation decreased in the insulin plus metformin-infused group compared with the insulin-only group, although the level of phosphorylation was still higher than in the control. In the muscle, the situation was similar: Akt phosphorylation was also stimulated by insulin by three-fold, but suppressed completely (returned to basal levels) in fish receiving insulin plus metformin. Finally, plasma (bovine) insulin levels were ~1 ng/ml in fish receiving insulin pumps, and plasma metformin levels ~50 μg/ml in those implanted with metformin pumps with no significant differences among the groups.

DISCUSSION

The glucose intolerance described in carnivorous rainbow trout reflected by persistent hyperglycemia in fish receiving glucose administration is still in debate. Although in trout, insulin has a hypoglycemic action (20) and metformin has an efficient antihyperglycemic action (29), the underlying mechanism remains to be clarified, and the contribution of the different peripheral tissues is still to be evaluated. Using osmotic pumps for chronic infusions, we combined a hypoglycemic hormone (insulin) with an antihyperglycemic drug (metformin) to check whether glucose utilization and tolerance is improved in rainbow trout, as it occurs in both type I and II diabetic patients (3, 34).

Glucose homeostasis after glucose challenge in trout under chronic saline, insulin, or metformin infusion. After 4 days of saline pump implantation, fasted trout subjected to glucose challenge exhibited hyperglycemia (by twofold) 6 h after the glucose injection. This is the first attempt in fish to assess glucose tolerance at the molecular level concomitantly in the two main organs involved in glucose homeostasis: liver and muscle. Actually, we found changes in hepatic mRNA levels of proteins involved in glucose transport, storage, and utilization that could contribute to a more efficient adaptation to increased glycemia. In contrast, the only pathway that seems not to be regulated directly by glucose at the molecular level is the de novo glucose production, whose actors (mRNA levels of FBPase and PEPCK) remain unaffected and surprisingly not inhibited by glucose, as observed earlier in trout fed with high-carbohydrate diets (27, 28). Although the lack of control of mRNA levels of gluconeogenic genes by glucose could be the reason for the persistent hyperglycemia noticed in trout after glucose administration, such contention is not supported by the important down-regulation of G6Pase mRNA levels observed in the present study. In fact, together with the increased GK mRNA levels, these results show an efficient regulation of the glucose/glucose 6-phosphate cycle at the molecular level in trout, as previously suggested by Moon (21). In the muscle, glucose storage as glycogen was also increased significantly (nine-fold induction) by glucose administration, although the mRNA levels of proteins involved in glucose transport and utilization remain unaltered, suggesting that both pathways could be regulated by glucose in different ways. Overall, the changes observed in the present study suggest an efficient glucose use and storage in peripheral tissues of rainbow trout after a glucose load, which do not agree with the profile of rainbow trout as a glucose-intolerant animal (40). Then, our results do not support the idea of weak peripheral glucose utilization in the liver and muscle as responsible for the prolonged hyperglycemia in this species after feeding carbohydrates (21).

When trout were implanted with insulin pumps, improved utilization of the injected glucose could be expected due to the chronic hormone infusion. Indeed, despite the fact that there was globally lower glycemia in insulin-infused fish than in saline-infused group, glycemia after glucose injection increased in insulin-implanted fish from 2.0 to 4.7 mM (difference = 2.7 mM), while in the control group, it increased from 3.7 to 8.1 mM (difference = 4.4 mM). This reduced increase in plasma glycemia in insulin-implanted fish suggests that the chronic insulin infusion allowed these animals to respond better to the glucose challenge. This glycemic profile could be explained by the fact that the glucose transport and utilization in muscle were enhanced by insulin, but not glucose storage, which was completely counteracted by insulin in clear contrast to the synergic effect of insulin and glucose seen in mammals (15). In addition, the increased glycogen storage (1.5 times more induction than in the control-infused group) and inhibition of glucose export (strong downregulation of G6Pase mRNA levels) indicate an increased storage of excess glucose and a reduction of its export to the bloodstream, probably improving the plasma glycemic profile.

Metformin is employed as a hypoglycemic drug specifically under hyperglycemic conditions (34), reducing glucose output in the liver and secondarily augmenting glucose uptake in the muscle (14). No hypoglycemia was observed in the present study in trout receiving chronically the drug and injected with saline solution alone. However, fish injected with glucose displayed the same level of hyperglycemia than those infused only with saline solution, which can suggest that metformin has no antihyperglycemic action in this species, in opposition to that previously showed in trout fed with carbohydrates plus

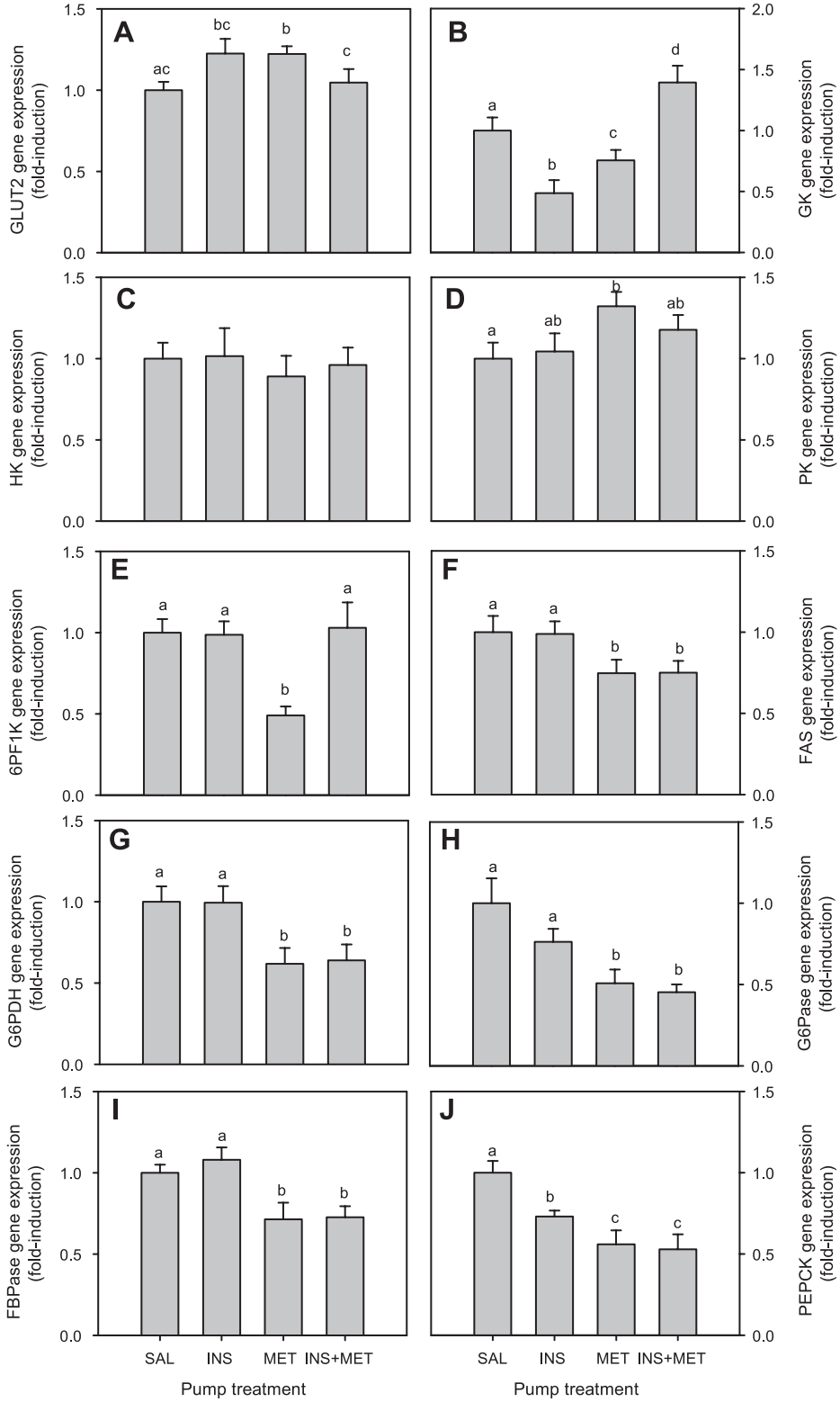

Fig. 2. Effects of intraperitoneal administration of glucose (6 h) in fish with an implanted pump for administering saline, insulin (1.4 IU/kg), metformin (80 mg/kg), or insulin plus metformin (4 days) on level of expression of mRNA encoding hepatic genes. Glucose facilitative transporter type 2 (GLUT2) (*A*), glucokinase (GK) (*B*), hexokinase (HK) (*C*), pyruvate kinase (PK) (*D*), 6-phophofructo-1-kinase (6PF1K) (*E*), fatty acid synthase (FAS) (*F*), glucose 6-phosphate dehydrogenase (G6PDH) (*G*), glucose 6-phosphatase (G6Pase) (*H*), fructose 1,6-bisphosphatase (FBPase) (*I*), and phosphoenolpyruvate carboxykinase (PEPCK) (*J*). mRNA levels were estimated using real-time RT-PCR. Results show variations between implanted groups in the hyperglycemic context, expressed as fold variation of the saline pump implanted group. Letters above the bars that differ indicate significant differences among groups ($P < 0.05$).

metformin (29). In this sense, as the main differences between our previous and present work are the nutritional status of fish and the duration of hyperglycemia, we suspect that these conditions could be certainly determinant for the hypoglycemic effect of the drug. In the present study, the fact that fish were food deprived for 6 days before the injection (only transient hyperglycemia) could make it more difficult for the drug to exert its effect on glycemia (if any) since other metabolic actions have probably been initiated in the fish to avoid the fall in plasma glucose levels due to the fasted condition. Actually, in the present study, mRNA levels of most proteins related to glucose transport and utilization in liver or muscle remained

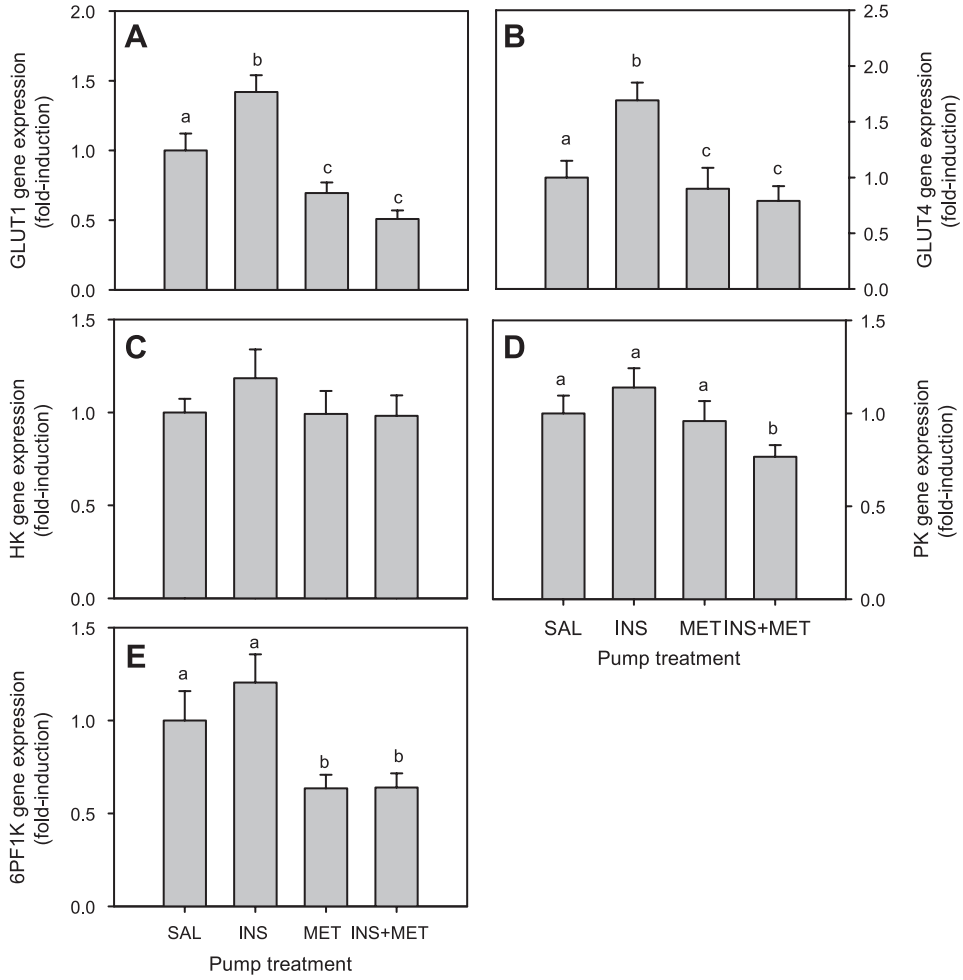

Fig. 3. Effects of intraperitoneal administration of glucose (6 h) in fish with an implanted pump for administering saline, insulin, metformin, or insulin plus metformin (4 days) on the level of expression of mRNA encoding muscle genes. Glucose facilitative transporter type 1 (GLUT1) (*A*), glucose facilitative transporter type 4 (GLUT4) (*B*), hexokinase (HK) (*C*), pyruvate kinase (PK) (*D*), and 6-phosphofructo-1-kinase (6PF1K) (*E*). mRNA levels were estimated using real-time RT-PCR. Results are expressed as fold variation of the saline pump-implanted group and were analyzed by one-way ANOVA followed by Student-Newman-Keuls comparison test. Letters above the bars that differ indicate significant differences among groups ($P < 0.05$).

unaffected by the drug after glucose injection. Despite this plasma profile, we found that metformin exerted its metabolic effects in both liver and muscle exclusively in glucose-injected fish. Then, muscle glycogen levels decreased after glucose injection in metformin-infused fish in opposition to the situation in the control group and in mammals (13), which could suggest that metformin is not only not improving insulin sensitivity in trout muscle, but is causing instead an insulin resistance. In contrast to these unexpected results in the muscle, we found decreased mRNA levels of lipogenic and gluconeogenic enzymes, which could result in more efficient suppression of these metabolic pathways in the liver. This mammalian-like metformin effect (38, 41) was also described in other fish species (7, 11), but surprisingly not in rainbow trout fed with carbohydrates plus metformin (29). The discordant effects of metformin on liver and muscle metabolism could explain the lack of differences in glycemia with respect to the control. In this sense, we found that even when the liver is responding well to the metformin action (favoring the return to basal glucose levels), the muscle does not, and the final effect could be reflected in the same degree of hyperglycemia as in the control groups.

Glucose homeostasis in trout receiving chronically insulin plus metformin infusion: interaction in hyperglycemic trout. In insulin plus metformin-infused trout, glucose led to significant hyperglycemia with respect to their control (saline injection), suggesting that there was no improvement of glucose homeostasis in these fish. Indeed, the increase in glycemia was higher (6.5 mM) in these fish than in those receiving only saline infusion (4.4 mM). This is not surprising if we compare these data with those of trout infused with metformin or insulin alone, in which no better glucose tolerance was observed either. However, even though saline-injected fish displayed hypoglycemia (as those infused only with insulin), trout receiving glucose injection show a metformin-like phenotype rather an insulin-like phenotype, with significant hyperglycemia. Then, it seems that metformin could be responsible for the suppressed insulin-like phenotype observed exclusively in glucose-injected trout. The hyperglycemia experienced by these fish as a consequence of metformin plus insulin infusion was completely unexpected, since type I diabetic patients receiving metformin added to insulin therapy show better insulin sensitivity. In fact, even hypoglycemia has been (rarely) reported in such patients (37) but never an insulin-suppressed effect due to metformin administration (33). Actually, as far we are aware, this is the first time that metformin has been found not only unable to return plasma glycemia to basal levels, but also to suppress the hypoglycemic effects of insulin, which make rainbow trout an attractive and original model for the study of glucose homeostasis.

This phenomenon was also observed at the metabolic level in liver and muscle of hyperglycemic trout. The lack of changes in mRNA levels of key gluconeogenic enzymes observed in insulin-treated fish was not found when insulin was

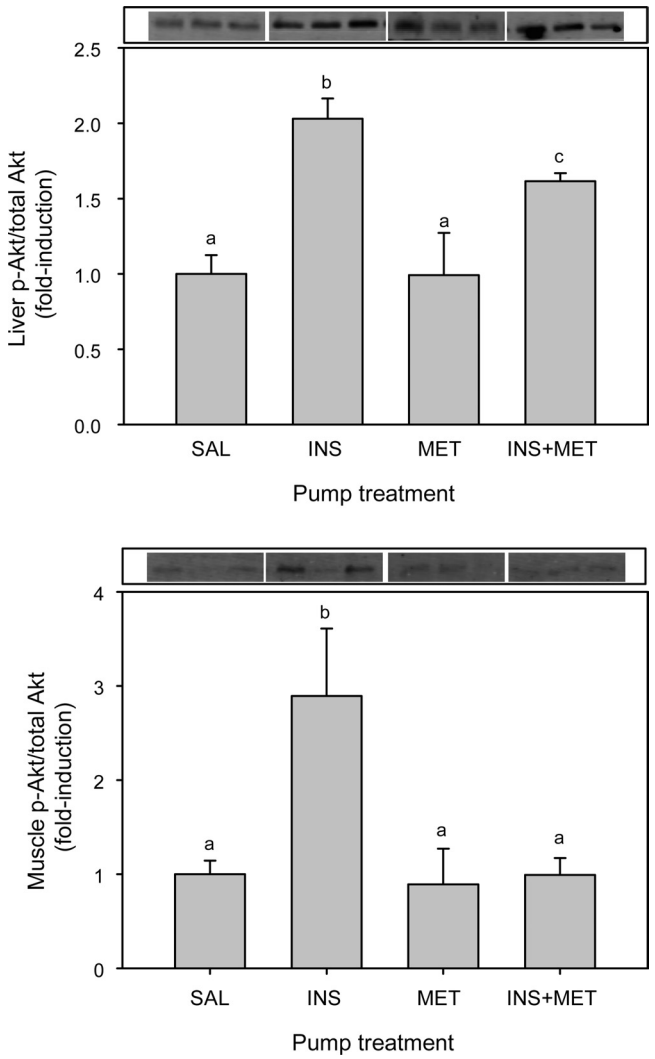

Fig. 4. Western blot analysis of the p-Akt-to-total Akt ratio in rainbow trout liver and muscle after intraperitoneal administration of glucose (6 h) in fish implanted with saline, insulin, or insulin plus metformin (4 days). The gel was loaded with 20 μg of total protein per lane. Results are expressed as means ± SE. ($n = 8$) and were analyzed by one-way ANOVA followed by Student-Newman-Keuls comparison test. Letters above the bars that differ indicate significant differences among groups ($P < 0.05$).

combined with metformin, but instead lower levels of mRNA levels of gluconeogenic enzymes, as in the metformin-infused group. The suppression of insulin action in fish receiving metformin plus insulin administration could be related with the diminished phosphorylation of Akt in this group, which, however, did not recover the basal levels observed in the saline-treated group. Similarly in the muscle, we found that the increased GLUTs mRNA levels due to chronic insulin treatment were counteracted by metformin. These results in trout muscle are opposite to those reported in mammalian L6 cells, in which insulin induced glucose transport was additive with that promoted by metformin (12). In the same sense, when metformin was combined with insulin, downregulation of mRNA levels of glycolytic enzymes was found in opposition to the lack of changes observed in the insulin-infused group. As a whole, these data suggest that glucose transport and utilization remain blocked in the muscle of trout chronically treated with insulin plus metformin, which together with the decreased glucose storage may explain the hyperglycemia experienced by these animals. As in the liver, we found suppressed Akt phosphorylation in the muscle of fish infused with insulin plus metformin compared with those receiving only insulin. Such an observation has never been reported in other species before and suggests that the predominant effect of metformin could be related with a suppression of the intracellular signaling of insulin. All together, we found that even when the glucose excess was normally stored and mRNA levels of gluconeogenic enzymes diminished in the liver of insulin plus metformin-implanted fish, the downregulation of mRNA levels of proteins involved in glucose transport, storage, and utilization in muscle does not allow trout to regulate correctly their plasma glucose levels, and they remain hyperglycemic.

Perspectives and Significance

We subjected carnivorous rainbow trout to chronic infusion of saline, insulin, metformin, and insulin plus metformin solutions followed by a glucose challenge. Although rainbow trout has been traditionally considered as glucose intolerant (40), we found no evidence that such behavior could be due to an impaired metabolic response at molecular level in liver or muscle. In this sense, as stated by Hemre et al. (10), it is possible that this picture was specifically limited to specific nutritional conditions (26) and not applicable to other physiological circumstances. Our data show that rainbow trout is a very useful animal model in which the antihyperglycemic drug metformin is unable to improve glucose homeostasis under hyperglycemic conditions, but, in fact, interacts negatively with insulin. This interaction found exclusively in glucose-injected fish was mainly due to the downregulation of mRNA actors involved in muscle metabolism (glucose transport, utilization, and storage), which become insulin resistant despite chronic insulin infusion. In the liver, the insulin resistance caused by metformin was less clear, with some of the hormone effects not affected by the drug, although the predominant metformin profile confirms the general suppression of insulin action in this fish species. In mammals, the molecular mechanisms by which metformin and insulin regulate glucose homeostasis when they are administrated together are still under debate (39). Although the molecular actors known to be involved in the metformin and insulin molecular response in mammals (e.g., the AMPK) are still to be fully characterized in fish, we cannot discard the possibility that in trout, some of them are not present or that, if present, they are not controlling glucose homeostasis in the same way. Further studies are needed to clarify the mechanism by which metformin is able to induce insulin resistance in rainbow trout.

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DISCLOSURES

No conflicts of interest are declared by the authors.

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