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SHORT COMMUNICATION

The five *glucose-6-phosphatase* paralogous genes are differentially regulated by insulin alone or combined with high level of amino acids and/or glucose in trout hepatocytes

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Abstract A recent analysis of the newly sequenced rainbow trout (*Oncorhynchus mykiss*) genome suggested that duplicated gluconeogenic *g6pc* paralogues, fixed in this genome after the salmonid-specific 4th whole genome duplication, may have a role in the setting up of the glucose-intolerant phenotype in this carnivorous species. This should be due to the sub- or neo-functionalization of their regulation. In the present short communication we thus addressed the question of the regulation of these genes by insulin, hormone involved in the glucose homeostasis, and its interaction with glucose and amino acids in vitro. The stimulation of trout hepatocytes with insulin revealed an atypical up-regulation of *g6pcb2* ohnologues and confirmed the sub- or neo-functionalization of the five *g6pc* genes at least at the regulatory level. Intriguingly, when hepatocytes were cultured with high levels of glucose and/or AAs in presence of insulin, most of the *g6pc* paralogues were up-regulated. It strongly suggested a cross-talk between insulin and nutrients for the regulation of these genes. Moreover these results strengthened the idea that *g6pc* duplicated genes may significantly contribute to the setting up of the glucose-intolerant phenotype in trout via their atypical regulation by insulin alone or in interaction with nutrients. These findings open new perspectives to better understand in vivo glucose-intolerant phenotype in trout fed a high carbohydrate diet.

Keywords Glucose-6-phosphatase · Duplicated genes · Insulin · Amino acids · Glucose

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Introduction

Rainbow trout, a carnivorous fish belonging to a high trophic level, is considered as a glucose-intolerant species thus displaying a persistent hyperglycemia when fed a high-carbohydrate diet [1–4]. Impairment of regulation of the gluconeogenic genes has been proposed to take part into the establishment of this glucose-intolerant phenotype in trout fed high-carbohydrate [5–7] or high-protein diet [8, 9]. Recently, a deep analysis of the newly sequenced genome of rainbow trout demonstrated that five duplicated genes encoding the glucose-6-phosphatase were retained after the salmonid-specific 4th whole genome duplication (Ss4R): *g6pca*, *g6pcb1.a*, *g6pcb1.b*, *g6pcb2.a* and *g6pcb2.b* [10]. Due to their atypical regulation by dietary carbohydrates, the *g6pcb2* ohnologues were proposed to contribute significantly to plasma glucose enrichment [10] and to the non-inhibition of the last step of the gluconeogenesis pathway. Indeed enzyme translated from these two genes may contribute to the release of glucose in blood via the establishment of a futile glucose/glucose-6-phosphate cycle together with glucokinase (which catalyze the phosphorylation of glucose into glucose-6-phosphatase, the first step of glycolysis). This study demonstrated that *g6pc* paralogous genes are differentially regulated by the nutritional status and dietary carbohydrates in vivo thus highlighting the importance of considering the fate of

duplicated genes after Ss4R to better understand the nutritional regulation of metabolism. This study provided evidences of a sub- or neo-functionalization of *g6pc* duplicated genes but did not allow to discriminate the role of nutritional hormones and/or specific nutrients in the atypical regulation of these genes. Consequently, completing this in vivo study by in vitro investigations will lead to clarify the role of specific nutritional actors such as insulin, carbohydrates or amino acids. Moreover, studying the regulation by hormonal and specific nutrients, such as glucose and amino acids (AAs), of these newly identified duplicated genes is now essential to provide knowledge concerning their related contribution to the glucose-intolerant phenotype in trout and also new answers to old questions. Actually, in analysis carried out before the sequencing of the trout genome, only two *g6pc*-related sequences were identified and expression data remained questioning when putting into perspective of enzyme activity results. For instance, insulin was shown to act at the transcriptional level to down-regulate *g6pc* mRNA level but had no effect on G6pc hepatic and gut activity [11]. In the same way, *g6pc* mRNA level was down-regulated by only one gluconeogenic dispensable AA (G-DAA), the alanine, whereas the enzyme activity decreased in trout fed a three G-DAAs substituted diets (alanine or aspartic acid or glutamic acid, [12]). The understanding of the regulation of the newly identified duplicated *g6pc* genes by Marandel et al. [10] may shed new light on this old questions. In this context, the present study aimed at analyzing the transcriptional effect of insulin alone or combined with high levels of glucose and/or AAs on *g6pc* and *gck* (glucokinase) paralogues in trout hepatocytes, a well-established and fully described system to analyzed the regulation of intermediary metabolism [13, 14].

Materials and methods

Data in this manuscript were obtained from the analysis of biological material used in articles previously published by Dai and collaborators [15, 16]. Primary hepatocyte cells were prepared as previously described by these authors. Prior to isolation of the liver cells, trout were left unfed for 72 h to empty the digestive tract and then facilitate in situ perfusion of the liver. At the time of the experiments, fish were anesthetized by placing them in water containing 60 mg l^{-1} aminobenzoic acid, and hepatocytes were isolated from three different individuals by in situ perfusion method described by Mommsen et al. [17]. Livers were excised and minced with a razor blade in modified Hanks' medium (136.9 mM NaCl, 5.4 mM KCl, 0.8 mM MgSO4, 0.44 mM KH2PO4, 0.33 mM Na2HPO4, 5 mM NaHCO3, and 10 mM HEPES) supplemented with 1 mM EGTA. After filtration and centrifugation (120 g, 2 min), the resulting cell pellet was resuspended three successive times in modified Hanks'medium [1.5 mM CaCl2 and 1.5 % defatted BSA (Sigma, St. Louis, MO)]. Cells were finally taken up in modified Hanks' medium supplemented with 1.5 mM CaCl2, 1 % defatted BSA, 3 mM glucose, MEM essential amino acids (1X) (Invitrogen, Carlsbad, CA), MEM nonessential amino acids (1X) (Invitrogen) and antibiotic antimycotic solution (1X) (Sigma) as the basic culture medium. Cell viability(98 %) was assessed using the trypan blue exclusion method (0.04 % in 0.15 M NaCl), and cells were counted using a hemocytometer. The hepatocyte cell suspension was plated in a six-well Primaria culture dish (Becton–Dickinson, Franklin Lakes, NJ) at a density of 3×10^6 cells/well and incubated at 18 °C. The culture medium was changed every 24 h over the 48 h of primary cell culture. Microscopic examination ensured that hepatocytes progressively reassociated throughout culture to form two-dimensional aggregates, in agreement with earlier reports [18, 19].

In the first experiment (described in [16] aiming at monitoring the effect of insulin on genes expression under basic cell culture conditions, 48 h-cultured cells were stimulated during 24 h with the medium containing 4×10^{-9} mol l^{-1} of bovine insulin (Sigma, St. Louis, MO, USA) corresponding to the post-prandial level of insulin irrespective of dietary carbohydrate level [20], and one fold (I*1AA) concentrated MEM essential and non-essential amino acids mixture, while the control cells maintained the basic culture medium (1AA but no insulin). The glucose concentration was maintained to 3 mM for this first experiment. For the second experiment (described in [15] involving different glucose and amino acids levels, 48 h-cultured cells were also stimulated with 4×10^{-9} mol l^{-1} of bovine insulin (Sigma, St. Louis, MO, USA), with low or high level of glucose (3 or 20 mM, respectively) [9, 21] and low or high level of amino acids (one-fold or four-fold concentrated amino acids, respectively). Cells collection and RNA extraction, cDNA synthesis and RT-PCR were performed as previously described [15, 16]. Primers used for amplifications of *g6pc* and *gck* genes were previously published by Marandel and collaborators [10]. For the first experiment (1AA vs. I*1AA), data were analyzed by a Kruskal–Wallis non-parametric test followed by a Tukey test as a post hoc analysis. For the second experiment (different amino acids and glucose levels), data were analyzed by a two-way ANOVA test followed by a Tukey test as a post hoc analysis. Data were analysed using the R software (v.3.1.0)/R Commander Package.

Ethic statement

The experiment was carried out in strict accordance with EU legal frameworks related to the protection of animals used for scientific purposes (Directive 2010/63/EU) and

guidelines of the French legislation governing the ethical treatment of animals (Decree no. 2001-464, May 29th, 2001). It was approved by the Direction Departementale des Services Veterinaires (French veterinary services) to carry out animal experiments (INRA 2002–36, April 14th, 2002). The INRA experimental station is certified for animal services under the permit number A64.495.1 by the French veterinary services, which is the competent authority.

Results and discussion

A recent study demonstrated that five *g6pc* genes were retained in the complex genome of rainbow trout after the Ss4R [10]. Before the sequencing of the rainbow trout genome [22] only two *g6pc*-related sequences were studied corresponding to the newly annotated *g6pca* (*e.g.,* primers named "*G6Pase*" in [7] and [6] or "*g6pase 2"* in [9] and [21]) and a conserved region between *g6pcb1* ohnologues by Marandel and collaborators (2015). Previous studies demonstrated that these two genes were down-regulated at the transcriptional level in primary hepatocytes stimulated by insulin and in liver after intraperitoneal injection of insulin [9, 21, 23]. Marandel et al. (2015) demonstrated that the five *g6pc* genes retained in the trout genome were differentially regulated by dietary carbohydrates, namely sub- or neofunctionalized. In order to better understand the hormonal regulation of the expression of these five paralogous genes, we thus further investigated their in vitro regulation by insulin (Fig. 1). Our results showed that *g6pca* mRNA level (Fig. 1a) was not significantly statistically affected by insulin stimulation while mRNA levels of *g6pcb1* ohnologues (Fig. 1b) were decreased. Surprisingly mRNA levels of *g6pcb2* ohnologues (Fig. 1c) displayed a dramatic increase similar to the one observed for *gck* ohnologues (Fig. 1d) and miming the expression pattern observed in vivo in trout fed a high carbohydrate diet [10]. These results first confirmed that *g6pc* genes were sub- or neo-functionalized at least at the regulatory level. Again the atypical up-regulation of *g6pcb2* ohnologues by insulin strengthened the idea that these two genes may contribute significantly to the non-inhibition of the last step of gluconeogenic flux and thus to the glucose-intolerant phenotype of trout as previously proposed [9, 10].

The regulation of glucose homeostasis involves mechanisms often depending on the cross-talk between nutritional and hormonal signals. Interactions between insulin and nutrients (glucose and AAs) has been shown to modify glycolytic and gluconeogenic genes expression in trout [11, 16, 23–25]. We therefore tested how *gck* and *g6pc* duplicated genes were regulated by glucose and/or amino acids in cultured hepatocytes stimulated by insulin.

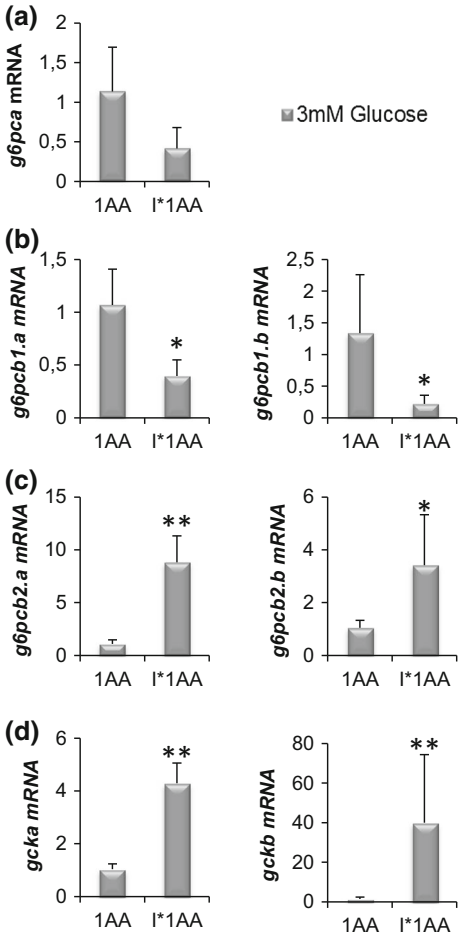

Fig. 1 Modulation by insulin of the regulation of *g6pc* (**a**–**c**) and *gck* (**d**) duplicated genes expression in trout hepatocytes. The experiment was conducted in 48 h-cultured cells. Cells were then stimulated with a medium containing 4×10^{-9} mol l^{-1} insulin (I) and one fold concentrated amino acids (I*1AA) while the control (1AA) cells were maintained in the basic culture medium (1AA without insulin). *One star*, $p < 0.05$; *two stars*, $p < 0.01$

We first showed that *gcka* mRNA level was not affected by the two nutrients (Fig. 2d) whereas *gckb* was unexpectedly down-regulated by high level of glucose (Fig. 2d). One hypothesis to explain this last down-regulation of *gckb* may be a sub- or neo-functionalization of *gckb* after the Ss4R regarding its regulation by high level of glucose in vitro.

Concerning gluconeogenic genes, our results showed that *g6pcb1.a* mRNA level was not affected by the two nutrients (Fig. 2b). Noteworthy mRNA level of *g6pcb1.a* was neither modified by dietary carbohydrate in vivo [10]. Other *g6pc* paralogous genes should be divided into two categories regarding the inhibitory or stimulatory effect of glucose and/or AAs on insulin regulation. In the first group including *g6pca* (Fig. 2a) and *g6pcb1.b* (Fig. 2b), high level of glucose overcame the inhibitory action of insulin and led to an increase in mRNA levels of these two genes.

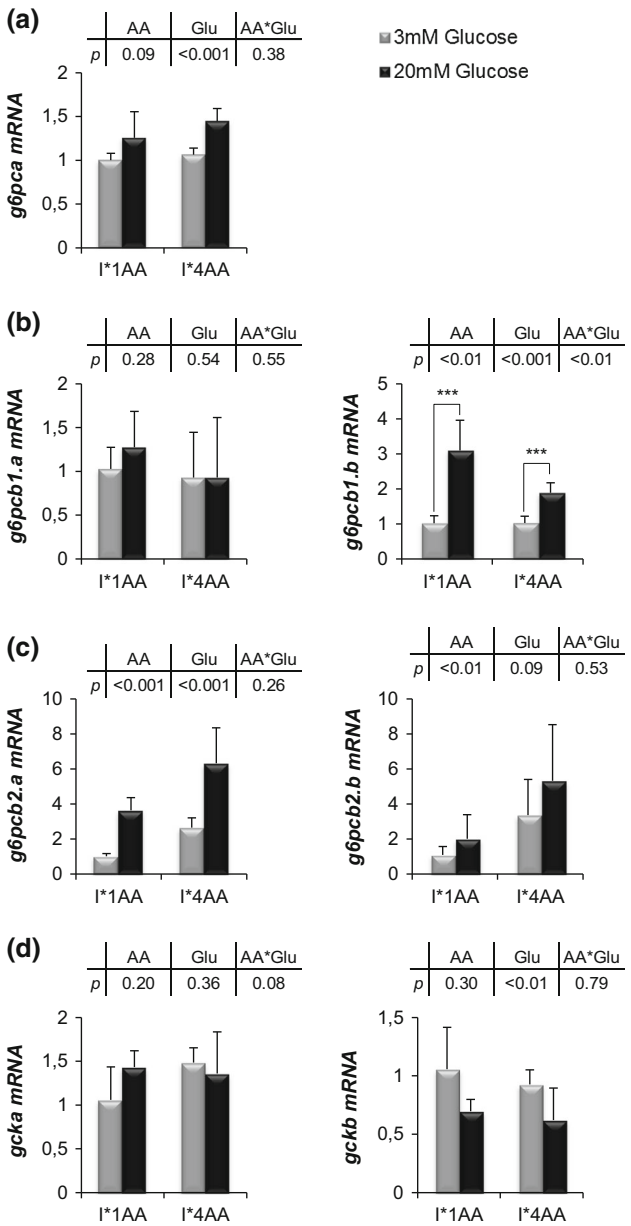

Fig. 2 Modulation by different glucose (Glu) and amino acids (AAs) levels of the regulation of *g6pc* (**a**–**c**) and *gck* (**d**) duplicated genes expression in trout hepatocytes. The experiment was conducted in 48 h-cultured cells. Cells were then stimulated with a medium containing 4×10^{-9} mol l^{-1} insulin (I), with low or high level of glucose (3 mM or 20 mM, respectively) and low or high level of amino acids (one-fold (1AA) or four-fold (4AA) concentrated amino acids, respectively)

This up-regulation by high level of glucose was opposite to the down-regulation previously observed in vivo [10] in trout fed a high carbohydrate diet. These findings suggested that the action of dietary carbohydrates on *g6pca* and *g6pcb1.b* genes may be indirect, interacting with other nutrients for instance. Interestingly, the effect of high glucose concentration on *g6pcb1.b* mRNA level was attenuated in cells cultured with a high concentration of AAs suggesting that there was a competitive interaction between these two nutrients in the regulation of this specific paralogue (Fig. 2b). In the opposite, high AAs level accentuated the stimulatory effect of insulin on the mRNA levels of *g6pcb2.a* and *g6pcb2.b* (Fig. 2c) leading to their increase. For these two paralogous genes, these data are in accordance with a previous in vitro study which demonstrated that high level of AAs attenuates insulin-mediated repression of gluconeogenic genes through the impairment of Akt activation, a critical node in insulin signaling pathway [16]. On the contrary, high level of amino acids contributed to reduce expression of *g6pcb1.b* under high concentration of glucose suggesting that *g6pcb1.b* was probably regulated by AAs under high glucose level through a different mechanism which requires more investigations to be elucidated. High glucose level also had an additional stimulatory effect on *g6pcb2.a* but independent from the one induced by high AAs level. This effect mimicked what happened in vivo in trout fed a high carbohydrate diet [10].

All effects induced by glucose and/or AAs described above, whether they were in agreement or opposite to the regulation induced by insulin, tipped the scale in favor of the non-inhibition of the last step of gluconeogenesis. In fact, when nutrients had an antagonistic effect to insulin they increased the mRNA level of genes down-regulated by insulin (i.e., *g6pca* and *g6pcb1.b*) while when they had an additional stimulatory effect they increased mRNA levels of genes that were still activated by insulin stimulation (i.e., *g6pcb2.a* and *g6pcb2.b*).

All these results confirmed that *g6pc* paralogues in trout were sub- or neo-functionalized as they were differentially regulated by insulin and nutrients. Such differences should be due to the DNA sequence per se, namely by the presence of different regulatory sequence fixing transcription factors. Indeed, insulin for instance can regulate gene transcription via several consensus insulin response sequences (IRSs) that mediate stimulatory or inhibitory effect of insulin [26]. A different chromatin environment between paralogues should also be responsible for differential regulation, epigenetic landscape being permissive or repressive. Also inhibitory effects on insulin action should be due to the induction of mTORC1/S6K1 feedback loop at least as a consequence of increased AA supply as previously proposed by Dai et al. [16]. In the contrary, mechanisms by which nutrients reinforce insulin action remain to be elucidated.

Finally, the present study strengthened the idea that specific nutritional phenotypes, such as the glucose-intolerant phenotype in trout, observed in teleost should not simply be due to an atypical regulation of metabolic pathway by dietary nutrients or hormones but well and

truly related to the structure of their genome and its complexity per se. Following this idea, the present findings confirmed that *g6pc* duplicated genes may contribute significantly to the non-inhibition of the last step of gluconeogenic flux and to the setting up of the glucose-intolerant phenotype in trout via their atypical regulation by insulin alone or in interaction with nutrients.

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