In vivo and in vitro insulin and fasting control of the transmembrane fatty acid transport proteins in Atlantic salmon (Salmo salar)

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Abstract

We have examined the nutritional and insulin regulation of the mRNA expression of transmembrane fatty acid (FA) transporters (FATP1 and CD36) together with the lipoprotein lipase (LPL), the cytosolic FA carrier FABP3 and mitochondrial FA-CoA and –carnitine carriers CPT1 and CPT2 in Atlantic salmon tissues and myocyte cell culture. Two weeks of fasting diminished FATP1, CD36 and LPL in adipose tissue, suggesting a reduction in FA uptake, while FABP3 increased in liver, probably enhancing the transport of FA to the mitochondria. Insulin injection decreased FATP1 and CD36 in white and red muscles while both transporters were up-regulated in the adipose tissue in agreement with the role of insulin inhibiting muscle FA oxidation and stimulating adipose fat stores. 48 h of serum deprivation in Atlantic salmon myotubes increased FATP1, FABP3 and CPT2 while CPT1 was diminished. In myotubes, insulin induced FATP1 expression but decreased CD36, FABP3 and LPL, suggesting that FATP1 could be more involved in the insulin stimulated FA uptake. Insulin increased the FA uptake in myotubes mediated, at least in part, through the relocation of FATP1 protein to the plasma membrane. Overall, Atlantic salmon FA transporters are regulated by fasting and insulin in in vivo and in vitro models.

Key words: FATP1, FAT/CD36, fatty acid binding protein (FABP3), lipoprotein lipase (LPL), carnitine palmitoyl transferase (CPT).
Introduction

Lipids are, jointly with proteins, the main energy source for carnivorous fish, such as salmonids, as is reflected in the high percentage of lipids in their feed (around 30% for Atlantic salmon). Although one of the main objectives in the development of aquaculture today is to maintain the benefits that the polyunsaturated fatty acid (FA) of fish flesh has for human consumption (14), little is known about the lipid metabolism regulation in fish muscle.

Lipid digestion, absorption, and plasma transport have been studied in fish (52). Dietary lipids can be accumulated, in some fish species, in the adipocytes surrounding myosepta or on those from visceral adipose tissue, leading to high levels of fat depots (36, 52). Fish have a relatively high capacity to use FA as metabolic fuel in skeletal muscle. Nevertheless, no studies are available on the regulation of the FA uptake by fish muscle cells despite its importance for FA metabolism. It has long been thought that entry of FA into cells occurs in an unregulated manner by diffusion (51), however, a facilitated kinetic transport of FA by rat adipocytes was reported later on (2). Nowadays, evidences in mammalian studies support a highly regulated protein-mediated system involved in FA transport into cells, that appears to be a key component in the regulation of FA metabolism (9). The first recognized FA transporter was called FATP1 (Fatty Acid Transport Protein 1) (46, 55) and more recently, other members of the family of FATP have been described in humans and rodents with different tissue localization and biochemical properties (42, 48, 50). The Fatty Acid Translocase (FAT/CD36) has also been characterized as FA transporter but it was initially described as a lipoprotein receptor and recently, it has been demonstrated to show activity as growth hormone-releasing peptide receptor (13, 44). In fish, FATP1 and CD36 presence has
been assessed only in Atlantic salmon (23, 28, 53) and very recently a facilitated kinetics of FA transport has been described in salmon hepatocytes (60). The expression of these FA transporters appears to be regulated by dietary replacement with vegetable oils (54).

In fish, insulin has been proved to be a hypoglycemic and pro-anabolic hormone (18, 37) with muscle being an important insulin target tissue. Therein insulin stimulated glucose transport, amino acid uptake and accelerates muscle protein synthesis (10, 11). Nevertheless, little is known about insulin regulation of lipid metabolism in fish, especially in muscle (45). In mammalian models, a reduction in FATP1 expression was found in human muscle after insulin treatment (7) while CD36 increased its expression with insulin in rat myocytes (12). Translocation capability of FATP1 and CD36 has been found in adipocyte and myocyte cell cultures (12, 24, 32, 49). Only one study on endocrine regulation of FA transporters has been reported in fish, in which an increased expression of CD36 was found after insulin stimulation in Atlantic salmon isolated hepatocytes (28).

Many species of fishes, including salmonids, have a natural fasting period in their normal annual life cycle, either because of spawning migration or as a result of low food availability. Fish respond to food deprivation by increasing the mobilization of the fat adipose tissue reserves to be used by other tissues such as muscle and heart (4, 6, 20, 25, 38). However, the biochemical mechanisms that lead those effects have not been defined.

Despite the importance of the plasma membrane FA transporters by themselves in the FA uptake, other important molecules are involved in the regulation of FA fluxes and it is of interest to analyze them to have a complete view of FA transporters functionality. LPL provides FA, from the TAG of lipoproteins (29), to the plasma membrane FA transporters. After
the internalization of the FA, they have to be carried across the cytoplasm by the cytoplasmic Fatty Acid Binding Proteins (FABP). Among other binding proteins, in the present study we have focused on FABP3 due to its important function in carrying the FA to the mitochondria (26) where the FA will be β-oxidized. To be taken up by the mitochondria, the FA rely on the activity of the carnitine palmitoyltransferase 1 and 2 (CPT1 and CPT2) (16, 35).

In the present study we investigated the regulation of transmembrane FA transporters (FATP1 and CD36) focusing in the effects of fasting and insulin in lipid metabolism relevant tissues, and in in vitro differentiated myotubes of Atlantic salmon. The expression of other important genes related to FA uptake and β-oxidation has also been analyzed. Furthermore, FA uptake rate and immunofluorescence against FATP1 in salmon myocytes after insulin stimulation were performed to elucidate its possible mechanisms of action.
Materials and methods

Fasting and insulin injection trials. For the fasting trial, 24 animals (Atlantic salmons, Salmo salar L, 1758) of approximately 128 ± 19 g and mixed sex were distributed randomly in two groups (12 each) at Institute of Marine Research (IMR) research station, Matre, Norway. One of the groups was fasted for two weeks while the other was fed (last meal 6h before sampling) with a commercial diet twice a day. At the end of the fasting period, all fish were anesthetized and killed by medullar section. Tissue samples were extracted, frozen immediately in liquid nitrogen and stored at -80°C until RNA extraction. Insulin injection experiment was carried out in 24h fasted mixed sex fish with an average weight of 1352 ± 36 g. 18 animals were injected with PBS (1µL/g) intraperitoneally as a control group while other 18 fishes were injected with PBS (1µL/g) containing porcine insulin at a dose of 2.16 nmol/100 g body mass as described previously (41). After 4, 12 or 24 h, 6 fishes from each group were anesthetized and blood was extracted by caudal vein. Fish were killed, and after dissection, tissues (liver, visceral adipose tissue, white and red muscles) were immediately frozen in liquid nitrogen and stored at -80°C until RNA extraction. Plasmas were obtained from total blood by centrifugation (700g, 10min) and stored at -20°C

Atlantic salmon muscle cell culture. Fish with an average length of 5 cm were used for the myosatellite cell isolation, as described previously (57). Briefly, fishes were killed by decapitation and white myotomal muscle was excised under sterile conditions and digested subsequently by collagenase (0.2% w/v) and trypsin (0.1% w/v). Satellite cells were recovered by centrifugation and seeded in laminine-pre-coated plates. Cells were maintained at 13°C in L15 medium supplemented with 10% fetal
bovine serum, 2mM L-glutamine and 1% antibiotics for 4 weeks (myotube stage), when all the experiments were carried out.

**FBS fasting and insulin stimulation on Atlantic salmon myocytes.** Differentiated myocyte cells (4-weeks old, myotube stage) were maintained in serum-free L15 for 48 hours to simulate *in vitro* fasting condition for cells and compared to cells growing in standard medium (L15 supplemented with 10% FBS) for the same period of time. For the insulin effects experiments, after 3h in serum-free medium, cells were stimulated with porcine insulin at 1µM for 30 minutes or 1h. Control cells were collected after 3h without serum. At the end of the treatments, all cells were lysed using the RNeasy Mini kit lysis reagent (Qiagen, Valencia, CA, USA) to isolate RNA.

**FA uptake.** 4-week old Atlantic salmon myocyte cells (myotube stage) were maintained for 3h in serum-free medium before the experiment. Then, medium was changed for fresh serum-free medium for the control cells or for medium containing insulin at 1µM for the insulin stimulated cells for the next 30 minutes. After this, medium containing 30µM of oleic acid and 0.3µCi/mL of 14C-Oleic acid (NEC 317050UC, Perkin Elmer) was added. The oleic acid was in its salt form bind to BSA fatty acids free at a ratio of 2.7:1 (57). After 2h, cells were thoroughly washed with cold PBS and lysed with NaOH 0.5M for scintillation counting. An aliquot was kept for protein determination by Lowry method (31).

**Immunofluorescence.** Four myocyte treatment groups were investigated for FATP1 localization including pre-starved, starved and insulin-stimulated (for 30 min or 1h). At the end of the treatments cells were all fixed with 4% paraformaldehyde in PBS for 10 minutes at room temperature and after a wash with TBS (TrisBase 0.05M, 0.9% NaCl, pH 7.6) cells were blocked for 45 minutes at room temperature with PBS
supplemented with 5% milk and 0.1% Tween® 20. After this, a solution consisting on PBS 2% milk containing an anti-hFATP1 (MAB3304, R&D Systems, Minneapolis, USA) antibody at 1/100 dilution was added to the cells for overnight at 4°C. Next day, cells were washed extensively and secondary antibody (Alexa Fluor® 647 goat anti-mouse IgG2b, Invitrogen, California, USA) was added to the cells in PBS 2% milk at 1/200 dilution and maintained for 3h at room temperature. Then cells were washed three times with TBS, one of them containing DAPI (Invitrogen) for nucleus staining and Cell Mask™ Orange (WGA) (Invitrogen) for cellular superficies staining. The coverslips were mounted using ProLong® Gold mounting medium (Invitrogen). Microscopy was carried out on a Zeiss Axiovision Z1 equipped for structured illumination (Apotome). Cellular localization of FATP1 was investigated in 3D models of image stacks. Clipped lateral views were used to study cytosolic and membrane localization of FATP1 signal. Negative control was performed by skipping the primary antibody anti-FATP1.

**RNA extraction and cDNA synthesis.** Total RNA from Atlantic salmon tissues was extracted using Trizol reagent (Invitrogen; Life Technologies, Carlsbad, CA, U.S.A.) standard protocol after homogenization in a Precellys 24 (Bertin technologies, Montigny-le-Bretonneux, FRANCE). For the myocyte RNA isolation, RNeasy Mini kit (Qiagen) was used. Genomic DNA contamination, from tissues and cells, was eliminated by the DNase kit from Ambion (Ambion Inc., Austin, TX, USA.). Quality and integrity of the RNA were assessed with a NanoDrop® ND-1000 UV-Vis Spectrophotometer (for the 260/280 nm ratio) (NanoDrop Technologies, Wilmington, DE, USA) and the Agilent 2100 Bioanalyzer (for the RNA integrity number (RIN)) (Agilent Technologies, Palo Alto, CA, U.S.A.) using the RNA 6000 Nano LabChip® kit (Agilent
Technologies). The RT reactions were run in duplicates for tissues and in triplicates for cells on 96-well reaction plates with the GeneAmp PCR 9700 machine (Applied Biosystems, Foster City, CA, USA) using Multiscribe Reverse Transcriptase (50 U/μL) (N808-0234, Applied Biosystems). Two-fold serial dilutions of total RNA were made for efficiency calculations. A dilution curve was recorded using six serial dilutions (1000–31 ng) in triplicates. Total RNA input was 500 ng in each reaction for all genes. No template control (ntc) and RT-control (a duplicate RNA sample analysis where only the RT enzyme is left out) reactions were run for quality assessment for each assay and gene.

Q-PCR. cDNA (2.0 μL) from each RT reaction for all genes was transferred to a new 96-well reaction plate, and the real-time PCR was run in 20 μL reactions on the Light Cycler® 480 Real-Time PCR System (Roche Applied Sciences, Basel, Switzerland) and the resulting Cts were recorded. Real-time PCR was performed using SYBR Green Master Mix (Light Cycler 480 SYBR Green master mix kit, Roche) containing the FastStart DNA polymerase, and gene specific primers (Table 1) at 500 nM. The geNorm VBA applet for Microsoft Excel was used to determine a normalization factor from the three examined reference genes (β-actin, eF1α and ARP) used to calculate mean normalized expression for the target genes (56).

Statistical analysis. Data are expressed as means ± SEM. Statistical differences between experimental conditions and corresponding controls were analyzed by t-Test. Differences were considered statistically significant at $P<0.05$. 

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Results

Atlantic salmon fasting and insulin injection. The mRNA expression of the selected genes was not affected by two weeks of fasting in white muscle (Fig. 1A) but the mRNA expression of FATP1, CD36 and LPL decreased in fasted Atlantic salmon adipose tissue (Fig. 1B). FABP3 was up-regulated in the liver of these fish with respect to the control fed fish (Fig. 1C).

Insulin injection in Atlantic salmon effectively reduced plasma glucose levels in all time points as expected (Table 2). In white muscle of insulin injected fishes, a decrease in the expression of both transporters FATP1 and CD36 was observed but at a different time point (24 and 4 h respectively) (Fig. 2). Red muscle showed a reduction in the expression levels of FATP1, LPL and CPT-1 mRNA at 12 h after insulin injection, while CD36 was reduced at 4 h and its expression was recovered after 12 h (Fig. 3). On the other hand, FATP1 and CD36 mRNA levels were up-regulated significantly in the visceral adipose tissue of the insulin injected fish at 4 and 12 h for FATP1 or only after 12 h in the case of CD36 (Fig. 4). Also, FABP3 and LPL showed a clear increase in their mRNA expression at both 4 and 12 h after insulin injection in the adipose tissue of these fish. In the liver of the insulin treated fish a significant increment in the CD36 mRNA quantity at 4 h was observed (Fig. 5). At 12 h post-injection, an increased expression of LPL by insulin was found and this increase was maximal at 24 h after injection when there was also an induction of CPT-1 expression.

Serum deprivation and insulin stimulation effects on Atlantic salmon muscle cells. After 48 h without serum, the differentiated myocytes of Atlantic salmon showed increased mRNA levels of FATP1, FABP3 and CPT2 while the CPT1 mRNA presence was decreased (Fig. 6).
Insulin stimulation on Atlantic salmon myotubes led to a significant increase of the FATP1 mRNA transcripts levels after 1 h (Fig. 7A) while CD36 decreased at both 30 min and 1 h of stimulation (Fig. 7B). FABP3 and LPL mRNA expression was down-regulated by insulin after 1 h of stimulation (Fig. 7C, D). No changes were observed in the mRNA expression of CPT-1 and 2 (Fig. 7E, F).

*FA uptake by Atlantic salmon myocytes and FATP1 immunofluorescence.* FA uptake was assessed in 4-week old Atlantic salmon myotubes with or without previous insulin stimulation. After 2 h of uptake, the insulin treated cells showed an increased FA uptake with respect to untreated cells (15.03% above) (Fig. 8).

To check the mechanism by which the FA uptake was increased, immunofluorescence against FATP1 was performed in cells incubated with insulin for 30 minutes or 1h. In the pre-starved cells (Fig. 9A, B) FATP1 signal was mostly evident in the plasma membrane (indicated by an arrow) and also in the cytoplasm (indicated by an asterisk) but after 3 h without serum FATP1 signal in the cell membrane was decreased and translocated intracellularly (Fig. 9C, D). After 30 minutes with 1µM insulin relocation of the FATP1 signal towards plasma membrane started (not shown) and after 1h of stimulation (Fig. 9E, F) very little staining for FATP1 was found in intracellular location while most of it was located in the plasma membrane. Negative control was performed and no signal was observed at the used settings (Fig. 9A insert).
Discussion

FA s have to be taken up by the cells as first step prior to any intracellular metabolic pathway. Recently, the presence of two FA transmembrane transporters has been described in fish tissues (28, 53) although their endocrine and nutritional regulation is almost unknown (54). For the first time in ectotherms, insulin and fasting effects on FA transporter expression and FA uptake regulation by insulin in different tissues and in muscle cells are reported in the present study.

Fasting is known to increase the lipolysis rate in the adipose tissue of fish (4, 20, 25) reflecting the mobilization of reserves needed for survival. This catabolic situation was evidenced in the adipose tissue by diminishing the expression of the FA transporters (FATP1 and CD36) and LPL. A lower FA uptake by the adipose tissue could increase the FA availability for other tissues, such as muscle, to use them for oxidation in a situation of low nutrient availability and activity. Contrarily, an up-regulation of FATP1 was found in the adipose tissue of 48 h fasted rats (33) which could be related with a high metabolic turnover of FA in this tissue and even it has been hypothesized that it could facilitated the FA efflux from fat cells to other tissues during lipolytic conditions (33, 34). Thus, it seems that this mechanism is not present in Atlantic salmon. The down-regulation of LPL mRNA expression in Atlantic salmon adipose tissue is in agreement with the reduced LPL mRNA expression and activity in adipose tissue found after a fasting period in mammals (58) and in various fish species (6, 8, 30). A lower LPL expression during fasting fits well with the observed decreased expression of the FA transporters in adipose tissue, tending to preserve the FA to other tissues such as skeletal muscle or hearth. The higher expression of FABP3 in fasted Atlantic salmon livers, as occurs in mammalian models (21), suggest an increased FA influx to the
mitochondria that could have capital importance during natural periods of food deprivation to maintain the energy production for fish liver cells (26). An increased influx of FA to the mitochondria and consequently increased fatty acid catabolism linked to the unavailability of nutrients during fasting is likely to be one of the main causes for the reduction in the lipid content of fish fasted livers (38).

Insulin increased the mRNA levels of both FA transporters in vivo in Atlantic salmon adipose tissue but decreased their expression in white and red muscles, as reported for human muscle (7). In this study we have analyzed separately the white (fast, glycolytic muscle) and red (slow, highly oxidative) muscles to assess the possible distinct response to insulin in those two metabolically different muscles. In all tissues analyzed we can observe that the expression levels of some genes present variations in the PBS treated between sampling moments. This effect reflects the natural variation in the expression of those genes along a day period. High insulin levels are correlated with nutrient availability (18), i.e. after a food intake, to replenish tissue energy storages. To our understanding, lipids would be directed to be stored in the adipose tissue after insulin treatment. In agreement with this hypothesis, the expression of LPL, as a marker of lipid uptake, was increased in Atlantic salmon adipose tissue while decreased in white and red muscle after insulin administration. This is consistent with the role of insulin stimulating fat storage in adipose tissue and inhibiting FA oxidation in skeletal muscle (37, 45). Similar increases after insulin injection in the LPL mRNA expression and activity in adipose tissue of other fish confirm the present results in Atlantic salmon (5, 6). As in rainbow trout, LPL transcription of salmon adipose tissue is much more sensible to the action of insulin than red or white muscle (6). Nevertheless, it has to be taken into account that mRNA expression changes of the FA
transporters or LPL do not necessarily imply an increase in FA uptake capacity or LPL activity by the tissue. The up-regulated expression of LPL in the liver seems to promote the accumulation of lipids also in the liver of Atlantic salmon accordingly to the role of this tissue as lipid reservoir in a post-feeding state (52). In concordance, insulin injected fish livers present an increased expression of CD36 as previously reported in isolated hepatocytes of Atlantic salmon (28). However, no changes in FATP1 mRNA expression were found and that could indicate a more important role of CD36 in liver of Atlantic salmon rather than the FATP1 after insulin increase while, in the muscles and adipose tissue, both transporters could be regulated by this hormone.

Salmon muscle CPT2, from the inner mitochondrial membrane, do not seem to be regulated by insulin, at least in our experimental conditions. Insulin was able to decrease CPT1 levels only in red muscle, in agreement with its oxidative function, and not in white muscle. In contrast, a diminution of CPT1b expression has been described in trout white muscle after insulin injection but only after chronic treatment (43). Other authors (40) have observed an inhibitory effect of insulin in the expression of CPT1a in trout liver, not confirmed in the present study in salmon, although insulin dose was much high in trout experiment.

Myocyte mRNA expression was affected after both treatments, 48 h without serum and incubation with insulin. Transmembrane FA transporters increased in serum deprived Atlantic salmon myotubes although only the rise in FATP1 was significant. This observation argues in favor of an increased uptake of FA. However, that effect was not found in the in vivo experiment of fasting in Atlantic salmon white muscle. The two models, serum-deprived myocyte culture and white muscle from starved fish, are not equivalent and direct comparisons should be done with care.
One of the possible causes of the differences observed between *in vitro* and *in vivo* results could be in part related to the presence of other cell types in the muscle from insulin-injected fish, as intramuscular adipocytes, that could interfere in the response. FATP1 was down-regulated by insulin *in vivo* but not *in vitro*. The presence of high levels of insulin in the whole fish could be affected by many other factors than *in vitro* which would favor the up-regulation of the transporter in adipose tissue and not in muscle.

Regarding the cytosolic FA carriers studied, a high presence of FABP3 mRNA in the serum starved myotubes suggests a high influx to the mitochondria and it correlate with the high expression of CPT2 we have observed, although unexpectedly the expression of CPT1 decreased significantly contrarily to that found in muscle of fasted rats (22). A fine and complex equilibrium of the levels of CPT1 and CTP2 expression and their activity must be reached in order to direct the FA flux to either obtain energy or reduce metabolic expenses.

Insulin stimulation leads to an increased expression of FATP1 but a decrease in the expression of CD36 in salmon myotubes. Previous reports in mammals refer to an increased expression of CD36 in murine muscle cells (12, 47) and either an up or down-regulation of FATP1 in mouse and human muscle, respectively, by insulin has been reported (7, 24). Thus, it is not clear whether the regulation of these transporters is similar to mammals or not. It is considered that muscle CD36 is also located in the mitochondria and along with CPT1 contributes to regulate mitochondrial FA transport and oxidation. This role is in agreement with the decreased level of transcription after insulin treatment observed. Besides, contradictory results have been reported about the actions of each FA transporter and different approximations suggest that, in mammalian cells,
CD36 could be responsible of basal rates of FA uptake in muscle. However, FATP1 could be more involved in the insulin stimulated FA uptake and triacylglycerol synthesis in this tissue (9) which it appears to be also the case in Atlantic salmon. LPL expression was negatively regulated by insulin in salmon myotubes, as in mammalian cells (15, 17). In any case, these findings reflect the complex and tissue specific regulation of the FA transporters transcription present also in Atlantic salmon.

It is very well known that, in short term, insulin stimulate the FA uptake in myocytes and adipocytes in mammals by inducing the transporters translocation to the plasma membrane (9). The increment in oleic acid uptake after insulin stimulation we report here (15% above control, approximately), although statistically significant, was slightly lower than those found for mammalian muscle cells (30-50% over control, approximately) (1, 24, 39). Using in vivo models, a reduction in the FA plasma levels after insulin injection was reported in rainbow trout, gilthead sea bream and lamprey (5, 20, 27). This decrease in plasma FA could be in part associated to an increase in FA uptake by tissues but also may be related to a reduction of the lipolysis rate in adipose tissue by insulin (3, 4, 19). We have found, for the first time in fish cells that FATP1 protein signal translocates from an intracellular compartment to the plasma membrane after insulin-stimulation, similarly to that described in muscle, muscle cells and adipocyte cultures of mammals (24, 32, 39, 49, 59). Although more studies are needed to determine the implicated pathway in the translocation of the FA transporters in fish muscle cells, it seems clear that the Atlantic salmon myotube cells are capable to translocate the FATP1 to the plasma membrane to increase the FA uptake in response to insulin similarly as reported for mammalian cells.
In summary, insulin and fasting effects on FA transporters tissue expression and FA uptake regulation by insulin in muscle cells are reported in the present study for the first time in ectotherms. The FA transporters are regulated by fasting and insulin, not always in the same way as it is reported in mammals, but in concordance with the different physiological role of each tissue.

Perspectives and significance

In the present study we demonstrated that transmembrane transporters are hormonally and nutritionally regulated at transcriptional level in salmon. These findings can contribute to the better understanding of the control of lipid metabolism specifically in the main fat depot organs in salmonids, muscle and adipose tissue, and may help to identify gene markers for adiposity, potentially useful to obtain a good quality product for aquaculture. In this sense, the substitution of marine oils by plant oils in fish diets is increasing in fish production. Vegetable oils have a different fatty acid composition and these differences are believed to modify lipid metabolism and fatty acids content of the whole organism. Further studies are clearly need to fully elucidate the mechanisms for fatty acid transport and uptake in fish tissues which may help to achieve a tailored and healthy level of fatty acids in final aquaculture products for human consumption.
Acknowledgments

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FIGURE CAPTIONS

**Fig. 1.** Effects of fasting on the gene expression of FATP1, CD36, FABP3, LPL, CPT-1 and CPT-2 in white muscle (A), adipose tissue (B) and liver (C) of Atlantic salmon. Fish were fed (black bars) or fasted (white bars) for 2 weeks prior to sampling and the gene expression of different genes was determined by qPCR and normalized using the *geNorm* normalization factor obtained from the three examined reference genes (β-actin, eF1α and ARP). Values are expressed as mean ± SE (n=12). Asterisk (*) indicates significant differences between fed and fasted (*P*<0.05, t-Test).

**Fig. 2.** Effects of insulin injection on the gene expression of FATP1, CD36, FABP3, LPL, CPT-1 and CPT-2 in white muscle of Atlantic salmon. Fish were injected intraperitoneally with vehicle (PBS) (black bars) or insulin (2.16 nmol INS/100 g body mass) (white bars) and sampled after 4, 12 and 24 hours. The gene expression of different genes was determined by qPCR and normalized using the *geNorm* normalization factor obtained from the three examined reference genes (β-actin, eF1α and ARP). Values are expressed as mean ± SE (n=6). Asterisk (*) indicates significant differences between control and insulin injected (*P*<0.05, t-Test).

**Fig. 3.** Effects of insulin injection on the gene expression of FATP1, CD36, FABP3, LPL, CPT-1 and CPT-2 in red muscle of Atlantic salmon. Fish were injected intraperitoneally with vehicle (PBS) (black bars) or insulin (2.16 nmol INS/100 g body mass) (white bars) and sampled after 4, 12 and 24 hours. The gene expression of different genes was determined by qPCR and normalized using the *geNorm* normalization factor obtained from the three examined reference genes (β-actin, eF1α and ARP). Values are expressed as mean ± SE (n=6). Asterisk (*) indicates significant differences between control and insulin injected (*P*<0.05, t-Test).
Fig. 4. Effects of insulin injection on the gene expression of FATP1, CD36, FABP3, LPL, CPT-1 and CPT-2 in adipose tissue of Atlantic salmon. Fish were injected intraperitoneally with vehicle (PBS) (black bars) or insulin (2.16 nmol INS/100 g body mass) (white bars) and sampled after 4, 12 and 24 hours. The gene expression of different genes was determined by qPCR and normalized using the geNorm normalization factor obtained from the three examined reference genes (β-actin, eF1α and ARP). Values are expressed as mean ± SE (n=6). Asterisk (*) indicates significant differences between control and insulin injected (P<0.05, t-Test).

Fig. 5. Effects of insulin injection on the gene expression of FATP1, CD36, FABP3, LPL, CPT-1 and CPT-2 in liver of Atlantic salmon. Fish were injected intraperitoneally with vehicle (PBS) (black bars) or insulin (2.16 nmol INS/100 g body mass) (white bars) and sampled after 4, 12 and 24 hours. The gene expression of different genes was determined by qPCR and normalized using the geNorm normalization factor obtained from the three examined reference genes (β-actin, eF1α and ARP). Values are expressed as mean ± SE (n=6). Asterisk (*) indicates significant differences between control and insulin injected (P<0.05, t-Test).

Fig. 6. Effects of serum deprivation on the gene expression of FATP1, CD36, FABP3, LPL, CPT-1 and CPT-2 in in vitro differentiated myotubes of Atlantic salmon. Cells were maintained with (black bars) or without (white bars) serum (10% FBS) in the culture medium for 2 days prior to RNA extraction. The gene expression of different genes was determined by qPCR and normalized using the geNorm normalization factor obtained from the three examined reference genes (β-actin, eF1α and ARP). Values are expressed as mean ± SE (n=3). Asterisk (*) indicates significant differences between control and serum deprived (P<0.05, t-Test).
**Fig. 7.** Effects of insulin stimulation on the gene expression of FATP1 (A), CD36 (B), FABP3 (C), LPL (D), CPT-1 (E) and CPT-2 (F) in *in vitro* differentiated myotubes of Atlantic salmon. Cells were maintained in a serum-free medium for 3 hours before stimulated them with insulin at 1µM for 30 min or 1 h prior to RNA extraction. The gene expression of different genes was determined by qPCR and normalized using the geNorm normalization factor obtained from the three examined reference genes (β-actin, eF1α and ARP). Values are expressed as mean ± SE (n=3). Asterisk (*) indicates significant differences between control and insulin (P<0.05, t-Test).

**Fig. 8.** Effects of insulin stimulation on the FA uptake in *in vitro* differentiated myotubes of Atlantic salmon. Cells were maintained in a serum-free medium for 3 hours before being stimulated them with insulin at 1µM for 30 min and FA uptake was performed as described in *Materials and Methods*. Values are expressed as mean ± SE (n=5). Asterisk (*) indicates significant differences between control and insulin (P<0.05, t-Test).

**Fig. 9.** *In vitro* studies on insulin stimulation on FATP1 in Atlantic salmon myotubes. FATP1 is shown in red, cell membrane (cell mask (WGA labeled)) green and nuclei white (DAPI) for all images. A) Cells incubated with normal serum (pre-starved) show strong staining for FATP1. Inset: negative control (Neg. ctr.). B) Transparency view describe membrane bound FATP1 as also shown by lateral view of the 3D image (inset on B). C) After 3 h of starving, a reduced staining for FATP1 is evident. D) A transparency model of the cells suggests cytosolic translocation as also shown by a lateral view (inset on D). E) After 3 hours of starvation, insulin treatment (1 h with 1 µM) increases FATP1 activity and translocation of
cytosolic FATP1 to the cell membrane (F and inset on F). Arrow indicated membrane location of FATP1. * indicated cytosolic location of FATP1.
Figure 1

(A) Normalised expression (RU) of FATP1, CD36, FABP3, LPL, CPT-1, and CPT-2 in Fed vs. Fasted conditions.

(B) Enlarged view showing significant differences marked with asterisks (*).

(C) Comparison of normalized expression (RU) for FATP1, CD36, FABP3, LPL, CPT-1, and CPT-2.
Figure 2
Figure 3

Bar charts showing normalised expression (RU) for different proteins:
- FATP1
- CD36
- FABP3
- LPL
- CPT-1
- CPT-2

The charts display data at 4h, 12h, and 24h, with control and INS conditions indicated.
Figure 4

- **FATP1**
  - Normalised expression (RU)
  - Control and INS
  - Significant difference indicated by asterisk (*)

- **CD36**
  - Normalised expression (RU)
  - Significant difference indicated by asterisk (*)

- **FABP3**
  - Normalised expression (RU)
  - Significant difference indicated by asterisk (*)

- **LPL**
  - Normalised expression (RU)
  - Significant difference indicated by asterisk (*)

- **CPT-1**
  - Normalised expression (RU)
  - Time points: 4h, 12h, 24h

- **CPT-2**
  - Normalised expression (RU)
  - Time points: 4h, 12h, 24h
Figure 5

Graphs showing normalized expression (RU) for different genes:
- **FATP1**
  - Control and INS groups with normalized expression values.
  - Significant differences indicated by asterisks.

- **CD36**
  - Control and INS groups with normalized expression values.
  - Significant differences indicated by asterisks.

- **FABP3**
  - Control and INS groups with normalized expression values.
  - Significant differences indicated by asterisks.

- **LPL**
  - Control and INS groups with normalized expression values.
  - Significant differences indicated by asterisks.

- **CPT-1**
  - Expression at 4h, 12h, and 24h with normalized expression values.
  - Significant differences indicated by asterisks.

- **CPT-2**
  - Expression at 4h, 12h, and 24h with normalized expression values.
  - Significant differences indicated by asterisks.
Figure 6

The figure shows a bar chart comparing the normalised expression (RU) of various genes: FATP1, CD36, FABP3, LPL, CPT1, and CPT2, under two conditions: Control and FBS fasted. The chart indicates that the expression levels are significantly different between the two conditions, with some genes showing a notable increase or decrease. The bars are accompanied by error bars to represent the standard error of the mean.

- FATP1: The expression is slightly lower in FBS fasted compared to Control.
- CD36: No significant difference is observed between the two conditions.
- FABP3: A significant increase in expression is observed in FBS fasted compared to Control.
- LPL: A slight decrease in expression is observed in FBS fasted compared to Control.
- CPT1: A significant increase in expression is observed in FBS fasted compared to Control.
- CPT2: No significant difference is observed between the two conditions.

The chart is annotated with asterisks (*) to indicate statistical significance.
Figure 7

- **FATP1**
- **FABP3**
- **CPT-1**
- **CD36**
- **LPL**

Graphs showing normalised expression with bars for Control, INS 30 min, and INS 1h, with asterisks indicating significant differences.
Figure 8
<table>
<thead>
<tr>
<th>Gene</th>
<th>GenBank accession number</th>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>Annealing temp (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FATP1</td>
<td>CA373015/AF023258</td>
<td>TGGGAGCTTTGTGGGTCAA</td>
<td>ACTTTCATGAGGCAGGATTGG</td>
<td>58</td>
</tr>
<tr>
<td>CD36</td>
<td>AY606034</td>
<td>TTTCCTGCTGCGCACCTT</td>
<td>GGTGCCGGTCATGAAAGATT</td>
<td>54</td>
</tr>
<tr>
<td>LPL</td>
<td>gi:14582900</td>
<td>GCCGACCTTTTGGATGCTG</td>
<td>ACGTCCACAAAGAGAGGATG</td>
<td>60</td>
</tr>
<tr>
<td>FABP3</td>
<td>AY509548</td>
<td>CACCGCTGACGACAGGAAA</td>
<td>TGCAGGAGGACATGCAACCA</td>
<td>60</td>
</tr>
<tr>
<td>CPT2</td>
<td>BG934647</td>
<td>TGCTCAGCTAGGCTTCCATATG</td>
<td>AGTGCTGAGGACACTGATG</td>
<td>54</td>
</tr>
<tr>
<td>CPT1</td>
<td>AM230810</td>
<td>CTTGGGAAGGGGCTGTGATC</td>
<td>CTTGGGAAGGGGCTGTGATC</td>
<td>60</td>
</tr>
<tr>
<td>ARP</td>
<td>AY255630</td>
<td>GAAAAATCATCCAATGGCTGTG</td>
<td>CTTGGGAGCAGGAGGACAGA</td>
<td>60</td>
</tr>
<tr>
<td>β-actin</td>
<td>BG933897</td>
<td>CCAAAGGCAAGGAGGAGAAG</td>
<td>AGGGGAACACTGGCCTGATG</td>
<td>60</td>
</tr>
<tr>
<td>eF1α</td>
<td>BG933853</td>
<td>TGGGCCCAGGATGCTGAC</td>
<td>CACGGGCAGGGCATG</td>
<td>60</td>
</tr>
</tbody>
</table>

Abbreviations: FATP, fatty acid transport protein; LPL, lipoprotein lipase; FABP, fatty acid binding protein; CPT, carnitine palmitoyl transferase; ARP, acidic ribosomal protein; eF: elongation factor
Table 2. Plasma parameters of Atlantic salmon of the insulin injection experiment

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Insulin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose (mM)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4 h</td>
<td>9.56 ± 1.48</td>
<td>4.57 ± 1.01*</td>
</tr>
<tr>
<td>12 h</td>
<td>9.25 ± 0.71</td>
<td>3.39 ± 1.00**</td>
</tr>
<tr>
<td>24 h</td>
<td>10.17 ± 0.53</td>
<td>3.36 ± 1.52**</td>
</tr>
<tr>
<td>TAG (mM)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4 h</td>
<td>2.59 ± 0.66</td>
<td>1.58 ± 0.07</td>
</tr>
<tr>
<td>12 h</td>
<td>2.49 ± 0.50</td>
<td>1.56 ± 0.07</td>
</tr>
<tr>
<td>24 h</td>
<td>1.66 ± 0.26</td>
<td>1.53 ± 0.38</td>
</tr>
<tr>
<td>Chol (mM)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4 h</td>
<td>6.55 ± 0.31</td>
<td>6.10 ± 0.37</td>
</tr>
<tr>
<td>12 h</td>
<td>5.78 ± 0.37</td>
<td>7.01 ± 0.26*</td>
</tr>
<tr>
<td>24 h</td>
<td>6.22 ± 0.22</td>
<td>7.15 ± 0.86</td>
</tr>
</tbody>
</table>

Values are the mean ± SEM. Values were analyzed by t-test (*p<0.05; **p<0.001). Abbreviations: Chol, cholesterol.