Hyperinsulinemia induces insulin resistance and immune suppression via Ptpn6/Shp1 in zebrafish

Rubén Marín-Juez, Susanne Jong-Raadsen, Shuxin Yang and Herman P Spaink

ZF-Screens BV, J.H. Oortweg 19, 2333 CH Leiden, The Netherlands
1Institute of Biology, Leiden University, Einsteinweg 55, 2333 CC Leiden, The Netherlands

Abstract

Type 2 diabetes, obesity, and metabolic syndrome are pathologies where insulin resistance plays a central role, and that affect a large population worldwide. These pathologies are usually associated with a dysregulation of insulin secretion leading to a chronic exposure of the tissues to high insulin levels (i.e. hyperinsulinemia), which diminishes the concentration of key downstream elements, causing insulin resistance. The complexity of the study of insulin resistance arises from the heterogeneity of the metabolic states where it is observed.

To contribute to the understanding of the mechanisms triggering insulin resistance, we have developed a zebrafish model to study insulin metabolism and its associated disorders. Zebrafish larvae appeared to be sensitive to human recombinant insulin, becoming insulin-resistant when exposed to a high dose of the hormone. Moreover RNA-seq-based transcriptomic profiling of these larvae revealed a strong downregulation of a number of immune-relevant genes as a consequence of the exposure to hyperinsulinemia. Interestingly, as an exception, the negative immune modulator protein tyrosine phosphatase nonreceptor type 6 (ptpn6) appeared to be upregulated in insulin-resistant larvae. Knockdown of ptpn6 was found to counteract the observed downregulation of the immune system and insulin signaling pathway caused by hyperinsulinemia. These results indicate that ptpn6 is a mediator of the metabolic switch between insulin-sensitive and insulin-resistant states. Our zebrafish model for hyperinsulinemia has therefore demonstrated its suitability for discovery of novel regulators of insulin resistance. In addition, our data will be very useful in further studies of the function of immunological determinants in a non-obese model system.

Introduction

Metabolic disorders (e.g. diabetes, obesity, and metabolic syndrome) have over the past three decades reached pandemic dimensions, affecting a large number of the world population (Wild & Byrne 2006, Lin & Sun 2010). Insulin resistance is a common aspect of a number of these metabolic pathologies, such as type 2 diabetes, and also a powerful parameter for predicting the incidence of cardiovascular diseases and cancer (Shanik et al. 2008). Hyperinsulinemia is the hallmark of insulin resistance in mammals. For instance, a twofold increase in plasma insulin levels can induce insulin resistance in mice, resembling the pattern in insulin-resistant patients with pathological obesity or type 2
diabetes who show similar increases in insulin levels (Shanik et al. 2008). As insulin resistance is common to a number of metabolic diseases of which the relationships are still poorly understood, it is difficult to study with respect to causal relationships and genetic factors that are involved. Current models used for the study of insulin resistance range from mutant rodents to obese monkeys (Shafrir 2010). Although these models reproduce the human situation with regard to obesity and insulin resistance, there still appear to be many conflicting results compared with the human situation, mainly due to the heterogeneity of the metabolic states where insulin resistance is observed (Harano et al. 2002, Kahn 2003, Steinberger & Daniels 2003).

The zebrafish (Danio rerio) is emerging as a model for deciphering the mechanisms underlying pathologies caused by an altered metabolism (Seth et al. 2013). Principles of energy expenditure and metabolism are evolutionarily conserved in metazoans (Schlegel & Stainier 2007). In addition, zebrafish larvae at 4 days post fertilization (dpf) have developed many functional organs (Elo et al. 2007), with the advantages inherent to the zebrafish larvae system as presented in many review papers (Lieschke & Currie 2007, Ali et al. 2011, Liu & Leach 2011, Meijer & Spanik 2011, Santoriello & Zon 2012, Driessen et al. 2013, Seth et al. 2013). We therefore set out to develop a non-obese zebrafish model for the study of insulin metabolism and its associated disorders. In this study, we report on the finding that 4 dpf zebrafish larvae are sensitive to human insulin and become insulin-resistant when treated with high doses of insulin. Moreover, by profiling the transcriptomic response of insulin-sensitive and insulin-resistant larvae, we further characterize the effects of hyperinsulinemia on the metabolism. At the transcriptome level, we observed a general downregulation of immune-relevant genes potentially mediated by protein tyrosine phosphatase nonreceptor type 6 (ptpn6).

PTPN6 (also known as SHP1) is expressed mainly in hematopoietic cells (Yi et al. 1992) and has been described as an important negative regulator of immune signaling pathways (An et al. 2008, Croker et al. 2008). In addition, it has been reported that PTPN6 may be involved in the development of insulin resistance and non-alcoholic fatty liver diseases in obesity (Xu et al. 2012, 2014a). Pioneering studies performed by Dubois et al. (2006) showed that PTPN6-deficient mice exhibited improved insulin sensitivity and glucose tolerance. Further studies by this group demonstrated that hepatocyte-specific Ptpn6 knockout (Ptpn6<sup>H-KO</sup>) mice were protected from hepatic insulin resistance and hepatocellular damage caused by diet-induced obesity (Xu et al. 2012, 2014a).

In this study, we made use of a previously described test system for Ptpn6 function in zebrafish, using reverse genetic approaches (Kanwal et al. 2013). We showed that knockdown of ptpn6 in zebrafish larvae interferes with the effects caused by hyperinsulinemia. In conclusion, using this non-obese vertebrate model, we were able to demonstrate that ptpn6 expression is stimulated by hyperinsulinemia, and that ptpn6 knockdown regulates key elements of the insulin pathway and also the leptin signaling pathway.

**Materials and methods**

**Fish maintenance**

WT zebrafish of the AB/TL strain were handled in compliance with the Local Animal Welfare legislation and maintained as described previously (Kimmel et al. 1995, Westerfield 2000). Embryos were grown at 28.5 °C in egg water (60 μg/ml Ocean Salts). During PBS/insulin injections, fish were kept under anesthesia in egg water containing 0.02% buffered 3-aminobenzoic acid ethyl ester (Sigma–Aldrich).

**Morpholino injections**

For knockdown of ptpn6, a morpholino oligonucleotide (Gene Tools, LLC, Philomath, OR, USA) targeting ptpn6, kindly donated by Dr A H Meijer, was injected (Kanwal et al. 2013). The morpholino (5′-ACTCATTCCTTACCGATGCGGACG-3′) was diluted to a concentration of 0.08 mM in 1× Danieau’s buffer (58 mM NaCl, 0.7 mM KCl, 0.4 mM MgSO<sub>4</sub>, 0.6 mM Ca(NO<sub>3</sub>)<sub>2</sub>, and 5.0 mM HEPES (pH 7.6)) and 1 nl was injected at the one-cell stage using a Femtojet injector (Eppendorf, Hamburg, Germany). Specificity of the morpholino was confirmed phenotypically and by RT-PCR (Supplementary Fig. 1, see section on supplementary data given at the end of this article).

**Insulin injections**

To inject PBS and human recombinant insulin (Sigma–Aldrich), 1 nl was injected into the caudal aorta of 4 dpf zebrafish larvae using a glass capillary (Supplementary Fig. 2, see section on supplementary data given at the end of this article).

**qPCR analysis**

RNA was isolated using TRIzol (Life Technologies). RNA samples were treated with RQ1 DNase (Promega) and reverse...
transcribed using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories B.V.), according to the manufacturers’ protocols. For the quantification of mRNA expression, qPCR was carried out using IQ SYBR Green Supermix (Bio-Rad Laboratories B.V.). The reactions were run in a iCycler Thermal Cycler (Bio-Rad Laboratories B.V.) under the following conditions: 2 min at 50 °C, 8 min at 95 °C, followed by 40 cycles of 15 s denaturation at 95 °C and 30 s at the corresponding melting temperatures, and a final melting curve of 81 cycles from 55 to 95 °C (0.5 °C increments for every 10 s). mRNA expression levels were normalized against the expression of ppial as a housekeeping gene. Primer sequences are presented in Supplementary Table 7, see section on supplementary data given at the end of this article.

Glucose measurements

Glucose measurements were done using a fluorescence-based enzymatic detection kit (Biovision, Inc., Mountain View, CA, USA) as described previously (Jurczyk et al. 2011).

RNA deep sequencing (RNA-seq)

Ten larvae per condition were homogenized in 1 ml of TRIzol reagent (Life Technologies), and total RNA was extracted according to the manufacturer’s instructions. RNA samples were treated with DNaseI (Life Technologies) to remove residual genomic DNA. RNA integrity was analyzed by Lab-on-a-chip analysis (Agilent, Amstelveen, The Netherlands). The average RNA integrity (RIN) value of the RNA samples was 9.7, with a minimum of 9.5. A total of 2 μg of RNA was used to make RNA-seq libraries using the Illumina TruSeq RNA Sample Preparation Kit v2 (Illumina, Inc., San Diego, CA, USA). The manufacturer’s instructions were followed with the exception of two modifications. In the adapter ligation step, 1 μl, instead of 2.5 μl, adaptor was used. In the library size-selection step, the library fragments were isolated with a double Ampure XP purification with a 0.7 x beads to library ratio (Beckman Coulter, Woerden, The Netherlands). The resulting mRNA-seq library was sequenced on an Illumina HiSeq2500 Instrument (Illumina, Inc.) according to the manufacturer’s instructions with a read length of 2 × 50 nucleotides. Image analysis and base-calling were done by the Illumina HCS version 2.0.12. Data analysis was performed using Genetiles Software (www.genetiles.com, W J Veneman, J de Sonnevile, K J van der Kolk, A Ordas, Z Al-Ars, A H Meijer and H P Spaïnk 2014, unpublished). False discovery rate (FDR)-adjusted P values were calculated based on the algorithm of Benjamini & Hochberg (1995). The raw RNA-seq data have been deposited in the NCBI GEO database under accession number GSE55836.

Western blot analysis

Whole, homogenized zebrafish larvae were prepared in lysis buffer (n=15/sample; 25 mM Tris–HCl (pH 7.6), 150 mM NaCl, 1% NP-40, 1% sodium-deoxycholate, and 0.1% SDS) with protease inhibitor (Roche Applied Sciences). The lysates were centrifuged at 12 000 g for 10 min at 4 °C and supernatants stored. Supernatants were incubated at 95 °C for 10 min. The protein concentration was determined using a BCA Protein Assay Kit (Thermo Scientific, Rockford, IL, USA). The extracted protein samples were subjected to SDS-PAGE and transferred onto nitrocellulose membranes (Bio-Rad). The membranes were blocked with 3% BSA in Tris-buffered saline with 0.1% Tween 20 for 1 h at room temperature. The membranes were then incubated with antibodies recognizing phosphorylated AKT (Cell Signaling Technology, Beverly, MA, USA; no. 9271, phospho-Akt Ser 473), total AKT (Cell Signaling, no. 9272), and Pan-actin (Cell Signaling, no. 4968) as a loading control overnight. After washing with Tris-buffered saline with 0.1% Tween 20, the membranes were incubated with the appropriate HRP-conjugated secondary antibodies (GE Healthcare, Chalfont St Giles UK) for 1 h and washed again. Immunoreactivity was detected by chemiluminescence (GE Healthcare). Finally, the bands were quantified by densitometry using ImageJ Software (National Institutes of Health, Bethesda, MD, USA). The bands were quantified by densitometry using ImageJ 64 Software (National Institutes of Health).

Results

Zebrafish that received human insulin showed inhibition of gluconeogenesis and transient hypoglycemia

In order to study whether zebrafish are sensitive to human insulin, we analyzed phosphoenolpyruvate carboxykinase 1 (pck1) expression levels because the transcription of this gene is well known to be inhibited by insulin (O’Brien & Granner 1990). For instance in mammalian systems, pck1 RNA levels are decreased after 20–40 min of insulin stimulation (Sasaki et al. 1984, O’Brien & Granner 1990). Zebrafish larvae at 4 dpf received injections of 1 nl of 100 nM human recombinant insulin into the caudal aorta (Supplementary Fig. 2A). qPCR analysis of pck1 expression was carried out in zebrafish larvae 0.5, 1, 2, and 4 h after injection (hpi) (Fig. 1A). The observed results confirmed that pck1 expression was significantly reduced in the presence of insulin at all the time points
studied (Fig. 1B). Next, to further confirm if insulin injection exerts the hypoglycemic effect observed in mammals, we carried out a time course experiment for measuring glucose levels of zebrafish larvae that received injections of insulin (Fig. 1A). Our results showed that, as in mammals, insulin administration leads to a fast reduction in free glucose levels from 15 to 45 min post injection, restoring normoglycemia from 2 hpi and onwards (Fig. 1C). Unfortunately, we failed to detect significant changes in Akt phosphorylation on the residue S473 30 min after injection, probably due to the fact that the analysis was performed at a whole-embryo level losing in this way sensitivity (Supplementary Fig. 2B).

Zebrafish larvae treated with a high dose of human insulin develop insulin resistance

The prolonged downregulation observed in pck1 expression, up to 4 hpi, together with the fact that larvae injected with 100 nM insulin may experience supra-physiological concentrations of the hormone, led us to propose the hypothesis that the larvae that received injections of insulin may be developing hyperinsulinemia-derived insulin resistance. To test this hypothesis, zebrafish larvae were administered human insulin as described earlier and at 4 hpi the same larvae received a second dose of insulin (Fig. 2A), while control individuals received injections of PBS at the same time points. Interestingly, after the injection of the second dose of insulin, zebrafish larvae exhibited transient hyperglycemia rather than the expected hypoglycemia (Fig. 2B). In addition, to confirm whether this is a dose-dependent effect, we carried out qPCR analysis of pck1 expression of larvae that had received injections of 1 nl of 10 nM, 100 nM, or 1 μM human insulin at 4 hpi. Our results indicated that at 10 nM, pck1 expression was not significantly reduced compared with results for control embryos, while embryos that received injections of 100 nM and 1 μM insulin
presented a significant downregulation of pck1 expression (Supplementary Fig. 3, see section on supplementary data given at the end of this article).

Next, to further confirm that zebrafish larvae at 4 hpi experienced insulin resistance, we analyzed Akt activity by measuring phosphorylation levels on residue S473. In agreement with the previous observations, larvae that received injections of 100 nM insulin at 4 hpi exhibited a significant reduction in Akt phosphorylation (Fig. 2C).

Overall, these results indicate that administration of high doses of human insulin to zebrafish larvae results in a loss of insulin sensitivity.

Transcriptomic profiling shows that key genes for the insulin metabolism and immune system are downregulated in hyperinsulinemic zebrafish larvae

To better characterize how the shift from an insulin-sensitive state to an insulin-resistant state may affect zebrafish larvae, we carried out an RNA-seq-based transcriptome analysis of zebrafish larvae that received injections of insulin at 0.5 and 4 hpi (Fig. 3A). Larvae that had received injections of PBS were used as controls and exhibited no significant changes in glucose levels during the experiment. Data (mean ± S.E.M.) are combined from four biological replicates (n=5 larvae/group). (C) Western blotting analysis of whole larvae at 4 hpi of PBS or 100 nM insulin was carried out as indicated in ‘Materials and methods’ section. Data (mean ± S.E.M.) are combined from four biological replicates (n=15 larvae/group) and expressed relative to their respective PBS control, which is defined as 1 (i). A picture of a representative immunoblot is shown (ii). *P<0.05 and **P<0.01. A full colour version of this figure is available at http://dx.doi.org/10.1530/JOE-14-0178.
metabolism (e.g. response to insulin stimulus, positive regulation of glucose import, and response to nutrient levels), innate immune response (e.g. regulation of cytokine production and upregulation of innate immune response), organ development (e.g. sensory organ development and embryonic skeletal system development), fatty acid metabolic process, digestive system process, and transmembrane transport (Fig. 3).

Interestingly, larvae at 4 hpi showed downregulation of insulin metabolism, confirming our previous observations, and also appeared to experience a general downregulation of immune-related processes (Fig. 3). Among DEGs involved in the insulin signaling pathway, all the genes known to play a central role in this pathway (insr, irs1, irs2, pik3cb, and pdk1) appeared to be downregulated at 4 hpi (Fig. 4). In addition, to further understand the effects on the innate immune system, we analyzed the expression of genes coding for important cytokines and for signal mediators and transcription regulators previously demonstrated to be relevant to the immune response (van der Vaart et al. 2012, Meijer et al. 2014). Strikingly, our data showed that all the genes studied in this context were downregulated at 4 hpi, except ptpn6, cxc3.1, cxc4b, and irf5 (Fig. 4). Furthermore, other genes known to have a relevant role during the defense response in zebrafish showed similar effects (Fig. 4).

Finally, we also studied genes important for various metabolic processes: genes involved in the leptin signaling pathway, gluconeogenesis and lipid metabolism. Overall, the observed effect on the expression of these genes confirmed the effect of insulin at 0.5 hpi, for instance on per2 and pck1 which are known to be rapidly regulated by insulin in mammals (O’Brien & Granner 1990, Tahara et al. 2011). The data also provide further evidence of the loss of insulin sensitivity at 4 hpi, as observed with lepb, lepr, gck,
and dpp4 reported to be dysregulated in insulin-resistant states (Wang et al. 2001, Jiang et al. 2011, Sell et al. 2013). In addition, the expression of il1b, cxcl-c1c, lepb, lepr, and pck1 was analyzed by qPCR to validate the RNA-seq data, the same effects on the expression of all the analyzed genes were detected by both techniques at 4 hpi of insulin (Supplementary Fig. 4, see section on supplementary data given at the end of this article).

Altogether, our data indicate that administration of a high dose of insulin inactivates the transcription of genes of importance for the immune system and downregulates the insulin signaling pathway and other metabolic genes. Therefore these results resemble gene expression patterns observed in insulin-resistant states in mammalian models (Ginsberg et al. 1976, Martin et al. 1983, Araki et al. 1994, Tamemoto et al. 1994, Patti & Kahn 1996, Withers et al. 1998, Boura-Halfon & Zick 2009).

**Knockdown of pttn6 prevents the downregulation of insulin and leptin signaling pathways and immune-relevant genes in hyperinsulinemic larvae**

One of the interesting observations from our transcriptomic profiling was that, despite the general immune suppression observed at the gene expression level, one of the few genes significantly upregulated at 4 hpi was pttn6. This gene is a well-known immune modulator playing a critical role as a negative regulator of the immune response (An et al. 2008, Croker et al. 2008). Importantly, PTPN6 has been described as a modulator of insulin signaling (Dubois et al. 2006), and recent findings have demonstrated its importance in the development of insulin resistance and non-alcoholic fatty liver diseases in diet-induced obesity (Xu et al. 2014a,b). Recently, Kanwal et al. (2013) have described a zebrafish model for pttn6 deficiency, in which
they observed that abrogation of this gene causes hyperactivation of the innate immune system, indicating that the piscine ortholog of \textit{ptpn6} may play the same role as its mammalian counterpart. In view of this, we proposed the hypothesis that long exposure to high plasma levels of insulin may stimulate \textit{ptpn6} expression, which in turn led to a dampening of the immune system and, at least partially, to the observed insulin-resistant phenotype. To test this hypothesis, we injected 100 nM insulin intravenously at 4 dpf in larvae that had previously received injections of the \textit{ptpn6} morpholino (Fig. 5A) which has been recently shown to efficiently abrogate \textit{ptpn6} expression (Kanwal et al. 2013).

At 4 hpi we analyzed (by qPCR) RNA expression of immune relevant genes, such as \textit{myd88}, \textit{traf6}, \textit{relb}, \textit{nfkb2}, \textit{il1b}, \textit{cxcl-c1c}, \textit{atf3}, and \textit{mfap4}, as well as genes important for leptin and insulin signaling pathways, namely \textit{lepb}, \textit{lepr}, \textit{insrb}, \textit{irs1}, and \textit{irs2}. Our results (Fig. 5B) show that the immune genes analyzed were not affected by insulin in the \textit{ptpn6}-deficient larvae, except in the case of \textit{il1b} and \textit{mfap4} where the same effects as detected by RNA-seq from WT larvae was observed. In addition, our data indicates that \textit{ptpn6} abrogation prevents the downregulation of all the metabolic genes analyzed, while the expression of \textit{lepr}, \textit{insrb}, and \textit{irs1} was significantly increased (Fig. 5C). Moreover, analysis of basal expression levels of \textit{il1b}, \textit{cxcl-c1c}, \textit{lepb}, and \textit{lepr} in \textit{ptpn6} morphants showed that \textit{il1b} and \textit{lepr} basal expression levels were similar to those of control embryos, while \textit{lepb} and \textit{cxcl-c1c} appeared to be significantly higher (Supplementary Fig. 5, see section on supplementary data given at the end of this article). We cannot exclude the possibility that the \textit{ptpn6} abrogation may prevent insulin resistance and immune suppression due to a basal overexpression of some genes (e.g. \textit{lepb} and \textit{cxcl-c1c}). Future in-depth studies would be needed to address this complex question.

Interestingly, a time course experiment (Fig. 6A) demonstrated that \textit{ptpn6} upregulation was not observed at earlier time points (Fig. 6B). Next, to further study whether \textit{ptpn6} morphants exhibit improved insulin sensitivity, we injected a second dose of insulin 4 hpi of the first dose and glucose levels were measured (Fig. 6A). Our results indicated that \textit{ptpn6} morphants presented a
significant reduction in glucose levels 30 and 120 min after the second injection, confirming that abrogation of ptpn6 improves the glycemic control of the larvae, therefore contributing to the prevention of the development of insulin resistance (Fig. 6C).

Discussion

In the present report, we describe a non-obese animal model for the study of hyperinsulinemia-derived insulin resistance. Using a newly developed method to administer human insulin intravenously into developed zebrafish, we demonstrate not only that human insulin is functional in zebrafish, but also that these zebrafish larvae become insulin-resistant when high concentrations of human insulin are administered by injection. Our findings indicate that this effect is mediated by stimulation of ptpn6 expression in the hyperinsulinemic scenario, leading concomitantly to immune suppression. Moreover, abrogation of ptpn6 was found to counteract the effects of high plasma levels of insulin on the transcription of key genes of the insulin and leptin signaling pathways as well as of the innate immune response.

After injection of 100 nM human insulin into zebrafish larvae at 4 dpf, pck1 transcription was rapidly inhibited and free glucose levels were significantly reduced at 15 min post injection, reaching normoglycemia again at 1 hpi, mimicking the effect in mammals (Sasaki et al. 1984, Barthel & Schmoll 2003, Seino et al. 2011). Next, the strong pck1 downregulation observed at 4 hpi led us to propose the hypothesis that larvae that had received injections of insulin...
could be experiencing insulin resistance caused by high plasma levels of insulin. In mammals, it is well established that insulin concentrations in the blood oscillate between post-absorptive periods, increasing again 4 h after each meal (Polonsky et al. 1988). In our case, a second insulin injection 4 h after the first injection did not cause transient hypoglycemia as observed after the first injection, instead we observed that these larvae showed transient hyperglycemia, consistent with other models of insulin resistance that present higher levels of plasma glucose (Shafrir 2010). However, it is worth mentioning that, despite not being statistically significant, at 2 hpi of the second insulin dose, glucose levels remained higher than in larvae that had received injections of PBS. This observation seems to contradict the observed downregulation of pck1 and g6pc at 4 hpi, as these genes have been typically described as mediating a rate-limiting step of gluconeogenesis. In consequence, hyperglycemic states in pathologies characterized by poor glycemic control (e.g. type 2 diabetes, obesity, and metabolic syndrome) have been thought to be associated with upregulation of both pck1 and g6pc expression. However, recent observations have challenged this paradigm. Samuel et al. (2009) demonstrated that expression of PEPCK and G6Pc was unaltered during fasting hyperglycemia using rodent models for diabetes. Moreover, in the same study, the authors showed that patients with type 2 diabetes did not exhibit any changes in hepatic PEPCK nor in G6Pc expression (Samuel et al. 2009). In addition, recently it has been shown that Pepck mutant mice exhibiting a 90% reduction in PEPCK protein levels only exhibit a 40% reduction in gluconeogenic flux (Burgess et al. 2007). Altogether, previous studies and the present data highlight the complex regulation of gluconeogenesis and indicate that further research focused on other less studied gluconeogenic genes (e.g. pyruvate carboxylase) should be carried out.

It is known that hyperinsulinemia can lead to insulin resistance in mammals by downregulating the mediators of the insulin signaling pathway (Kahn & Flier 2000), as shown by a number of in vivo studies where administration of high doses of insulin led to insulin resistance (Kobayashi & Olefsky 1978, Martin et al. 1983, Rizza et al. 1985). Supporting this notion, our data indicated that the transcription of genes coding for the key mediators of the insulin signaling pathway was inhibited, coinciding with the loss of insulin sensitivity and a decrease in Akt phosphorylation which is associated with insulin-resistant states (Tomas et al. 2002). Moreover, at this stage hyperinsulinemic larvae appeared to be immuno-suppressed at the transcriptional level, whereas the only immune modulator showing an increased expression was ptprn6. In mammals and zebrafish, ptprn6 has been reported to be a negative regulator of the immune response (Zhang et al. 2000, Tsui et al. 2006, Pao et al. 2007, An et al. 2008, Croker et al. 2008, Lorenz 2009, Kanwal et al. 2013) and a key determinant of development of insulin resistance and non-alcoholic fatty liver diseases (Xu et al. 2012, 2014a). We found that knocking down of ptprn6 interfered with the inhibitory effect observed on key transcription factors and signal mediators of the NF-κB pathway as well as on various cytokines in larvae that had received injections of insulin. It is worth mentioning that, although il1b expression was reduced after insulin injection also in ptprn6 knocked-down larvae, this is consistent with the observation that il1b expression was altered in ptprn6 morphants at 5 dpf and onwards, but not at 4 dpf (Kanwal et al. 2013). Besides this, we also observed that some genes identified as playing a role in innate immunity, such as mfap4, nos2b, cxcr3.1, cxcr4b, and irf5, were upregulated at 4 hpi, and after ptprn6 abrogation mfap4 was still induced by insulin. We cannot rule out the possibility that other immune modulators involved in particular tissue-specific innate immune processes are under-represented in our data set because in our model we focussed on major changes at the whole-embryo level. Future studies on dissected organs or FACS-sorted cells using tissue-specific labeled transgenic lines will be helpful to improve our understanding of which mechanisms contribute to the development of this immune dysregulation.

Studying the effect of ptprn6 abrogation on the insulin signaling pathway, we observed that morphant larvae administered insulin exhibited an increase in the expression of insrb, irs1, and irs2 (though not significant in the case of irs2). These results are in agreement with data from Ptpn6-deficient (Ptpn6<sup>me-v/me-v</sup>) mice, which displayed

![Figure 7](http://joe.endocrinology-journals.org/C209/DOI/10.1530/JOE-14-0178)

Figure 7

Model for hyperinsulinemia-induced immune suppression and insulin resistance via ptpn6. A full colour version of this figure is available at [http://dx.doi.org/10.1530/JOE-14-0178](http://dx.doi.org/10.1530/JOE-14-0178).
increased tyrosine phosphorylation of INSR, IRS1, and IRS2 and an enhanced insulin sensitivity (Dubois et al. 2006). Supporting this notion, ptptn6 morphants showed improved glycemic control, exhibiting hypoglycemia up to 2 h after administration of a second dose of insulin. This is consistent with the previous observations and confirms the central role of ptptn6 in mediating hyperinsulinemia-derived insulin resistance in zebrafish.

In addition, key members of the leptin signaling pathway, namely lepb, are known to be regulated by nutritional factors in zebrafish as in its mammalian ortholog (Gorissen et al. 2009), and lepr appeared to be downregulated in insulin-resistant larvae while ptptn6 abrogation was found to prevent this transcriptional inhibition.

Interestingly, it has been observed that stimulation of PTPN6 by insulin leads to JAK2 dephosphorylation, therefore interfering with the leptin signaling pathway (Kellerer et al. 2001). With these observations in mind, and knowing that animal models for insulin resistance with mutations in leptin or leptin receptor genes are immuno-suppressed it is tempting to propose the hypothesis that hyperinsulinemia may downregulate the insulin signaling pathway leading to insulin resistance via induction of ptptn6. Our results also indicate that the observed immuno-suppression may be due to a combination of factors, of which ptptn6 is exerting its role as a negative immune regulator by inhibiting the leptin signaling pathway that eventually contributes to the observed immune dysregulation (Fig. 7).

These observations raise an interesting point for discussion. Leptin-deficient rodents used as models for insulin resistance are hyperinsulinemic, insulin-resistant, and immuno-compromised (Meade et al. 1979, Chandra 1980, Zhang et al. 1994, Lord et al. 1998, Howard et al. 1999). Importantly, these observations are, in part, opposite to the human scenario where obese individuals that develop insulin resistance exhibit high leptin levels and an induction of inflammation (Gregor & Hotamisligil 2011). To circumvent this, high-fat-diet-induced obese mice are also used as a model as they develop insulin resistance and hyperinsulinemia, but still there are some discrepancies with the human situation. One of the major issues we face when studying insulin resistance is that this is a state associated with a number of pathophysiology, and in consequence a multifactorial disease, making it difficult to study using animal models. In this light the main contribution of our model to the existing ones is that it complements them as it allows the study of insulin resistance in a non-obese animal, helping in this way to clarify the mechanisms by which insulin exerts its effect and more importantly how this can trigger insulin resistance.

In summary, in the present study, we provide a new tool for the study of insulin metabolism and insulin resistance. Taking advantage of the zebrafish model, in which we can easily obtain and handle large numbers of non-obese individuals with fully functional organs, we have demonstrated that zebrafish larvae are sensitive to human insulin. Importantly, we showed how i.v. injection of a high dose of insulin leads to insulin resistance and immune suppression. Moreover, we showed that modulating ptptn6 expression interferes with the effects caused by hyperinsulinemia, preventing the downregulation of insulin and leptin signaling pathways and blocking the immune dysregulation. As mentioned by Kanwal et al. (2013), ptptn6 regulation of transcription and its relation with immune suppression via the glucocorticoid pathway is still poorly understood. Future studies will be needed to elucidate the even more complex role that ptptn6 plays regulating the transcription of the metabolic and immune signaling pathways mentioned in this study.

In conclusion, we present a new hyperinsulinemia animal model that complements the existing rodent models, allowing the study of insulin resistance in a non-obese state, and propose ptptn6 as a key mediator triggering hyperinsulinemia-derived insulin resistance and immune suppression. This important connection between insulin regulation and the immune system can now be studied further in the various infection and cancer models that have been recently developed for zebrafish.
Acknowledgements

The authors thank Dr Annemarie H Meijer for the gift of the ptpn6 morpholino. They deeply express their indebtedness to Drs Josep V Planas, Annemarie H Meijer, and Anna-Pavlina Haramis for critical reading of the manuscript. They thank Davy de Witt, Ulrike Nehrdich, and Laura van Hulst for fish caretaking and Hulya Ozupek, Drs Hans Jansen, and Ron Dirks (ZS-Screens B.V.) for assistance with RNA-seq analysis. They also want to thank Dr Jan de Sonneville and Kees-Jan van der Kolk for making the Genetiles Software available before publication and to Vanessa Jimenez-Amiliburu for her assistance with the western blotting analysis.

References


Steinberger J & Daniels SR 2003 Obesity, insulin resistance, diabetes, and cardiovascular risk in children: an American Heart Association scientific statement from the Atherosclerosis, Hypertension, and Obesity in the Young Committee (Council on Cardiovascular Disease in the Young) and the Diabetes Committee (Council on Nutrition, Physical Activity, and Metabolism). Circulation 107 1448–1453. (doi:10.1161/01.CIR.0000060923.07573.F2)

Received in final form 30 May 2014
Accepted 5 June 2014
Accepted Preprint published online 5 June 2014