

Larval zebrafish as a model for glucose metabolism: expression of phosphoenolpyruvate carboxykinase as a marker for exposure to anti-diabetic compounds

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Abstract

The zebrafish model system is one of the most widely used animal models for developmental research and it is now becoming an attractive model for drug discovery and toxicological screening. The completion of sequencing the zebrafish genome and the availability of full-length cDNAs and DNA microarrays for expression analysis, in addition to techniques for generating transgenic lines and targeted mutations, have made the zebrafish model even more attractive to researchers. Recent data indicate that the regulation of glucose metabolism in zebrafish, through the production of insulin, is similar to mammalian models, and many of the genes involved in regulating blood glucose levels have been identified in zebrafish. The data presented here show that adult zebrafish respond to anti-diabetic drugs similarly to mammalian models, by reducing blood glucose levels. Furthermore, we show that the expression of phosphoenolpyruvate carboxykinase (PEPCK), which catalyzes a rate-limiting step in gluconeogenesis and is transcriptionally regulated by glucagon and insulin, is regulated in larval zebrafish similarly to that seen in mammalian systems, and changes in PEPCK expression can be obtained through real-time PCR analysis of whole larval RNA. Taken together, these data suggest that larval zebrafish may be an appropriate model for the examination of glucose metabolism, using PEPCK as an indicator of blood glucose levels.

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Introduction

The zebrafish model system is becoming one of the most widely used animal models for developmental research because of its fecundity and its genetic and physiological similarities to mammals (Vascotto *et al.* 1997, Parng *et al.* 2002). These advantages are leading to the use of the zebrafish model in drug discovery and toxicological screening. The zebrafish embryo is particularly suited to high-throughput screening, due to the small size and optical transparency of the embryo. The utility of the zebrafish as a model organism is further enhanced by the completion of sequencing the zebrafish genome and the availability of full-length cDNAs and DNA microarrays for expression analysis. In addition, techniques for generating transgenic lines, targeted mutation, and nuclear transfer have made the zebrafish model even more useful to researchers.

Hyperglycemia in type II diabetes results from an inability of insulin to control gluconeogenesis (Stumvoll *et al.* 2005). When glucose is available in the diet, insulin is produced by the pancreas, and gluconeogenesis is inhibited through the downregulation of genes involved in the pathway. In the absence of glucose in the

bloodstream, gluconeogenesis is induced by the action of glucagon. Insulin and glucagon are produced in the β -cells and α -cells of the pancreas respectively. As in mammals, the zebrafish pancreas is also comprised of two types of glandular tissues, each of which carries out essential physiological functions (Gnugge *et al.* 2004). The endocrine tissue is critical for the regulation of glucose metabolism through secretion of insulin, somatostatin, and glucagon directly into the bloodstream. The exocrine portion of the pancreas produces digestive enzymes, including trypsin and amylase, which are carried to the digestive track. Hyperglycemia in type II diabetes has a complex etiology resulting from defects in insulin production and signaling, changes in insulin sensitivity of target tissues, as well as from increased hepatic gluconeogenesis (see Petersen & Shulman 2006 for review). The genes of zebrafish insulin and glucagon, as well as other important proteins in the regulation of glucose metabolism, have been identified and demonstrate similar regulation patterns and activity as seen in mammalian counterparts (Gnugge *et al.* 2004, Yee & Pack 2005, Yee *et al.* 2005).

Phosphoenolpyruvate carboxykinase (PEPCK) catalyzes the rate-limiting step in gluconeogenesis, i.e. the conversion of oxaloacetate to phosphoenolpyruvate

(Quinn & Yeagley 2005). Because PEPCK mRNA expression is transcriptionally modified by several glucoregulatory hormones, including insulin and glucagon, it is an excellent marker to investigate the effects of compounds on the regulation of glucose levels. Glucagon stimulates PEPCK through activation of cyclic AMP (Leahy *et al.* 1999, Yamada *et al.* 1999, Herzig *et al.* 2001, Liu *et al.* 2001, Monroy *et al.* 2001, Waltner-Law *et al.* 2003). In addition, PEPCK gene expression is also induced by glucocorticoids, all-trans retinoic acid, and thyroid hormone (Stafford *et al.* 2001, Waltner-Law *et al.* 2003), whereas both glucose and insulin inhibit PEPCK expression (O'Brien *et al.* 2001).

To examine the potential for zebrafish as a model for the regulation of glucose levels, we exposed adult and larval zebrafish to known anti-diabetic compounds. Our data show that blood glucose levels of the adult zebrafish are reduced following exposure to glipizide, an anti-diabetic drug. Furthermore, our data show that PEPCK expression in the larval zebrafish is regulated similarly to that of mammalian systems, with the expression being enhanced by cAMP/dexamethasone and all-trans retinoic acid and significantly reduced by exposure to anti-diabetic drugs, glipizide, metformin, and rosiglitazone.

Materials and methods

Fish maintenance

The zebrafish (AB strain) were maintained in a recirculating system (Aquatic Habitats) in a cycle of 14 h light:10 h darkness. Embryos were collected at 3 h post-fertilization (hpf) for experiments.

Exposure of adult zebrafish

Adult zebrafish were treated transdermally with 25% glucose in Me₂SO with or without 1.5 mg/kg glipizide (Sigma–Aldrich) dissolved in Me₂SO. Blood was collected 5, 16, and 24 h post-treatment for the analysis of blood glucose levels. Blood glucose levels were measured with OneTouch Ultra (Johnson & Johnson, New Brunswick, NJ, USA) blood glucosimeter.

Exposure of larval zebrafish

Zebrafish embryos were collected and allowed to develop until 96 h post-fertilization (hpf). This age was selected as the yolk sac is diminishing which simulates a starved state. At this time, the collected embryos had either hatched naturally or were mechanically dechorionated to allow for efficient transdermal exposure. The dechorionated embryos were exposed to the selected compounds for 48 h at 28–30 °C. Treatment concentrations were as follows: cyclic AMP (cAMP: 100 µM;

Sigma), dexamethasone (DEX: 250, 500, 1000 nM; Sigma), glucose (GLU: 40 mM; Sigma), metformin (MET: 10 µM; Sigma), glipizide (GLIP: 100–250 µM; Sigma), all-trans retinoic acid (atRA: 10 mM; Sigma), and rosiglitazone (ROSI: 1 µM; Sigma).

RT-PCR

Embryos collected for RNA isolation were snap frozen and total RNA isolated using TRIzol (Invitrogen) according to the manufacturer's instructions. The isolated RNA was DNase treated (DNA-free kit, Ambion, Austin, TX, USA) to remove genomic DNA contamination and cDNA prepared using the iScript cDNA Synthesis Kit (Bio-Rad). For fluorescent detection of PCR products, reactions containing template and specific primers were amplified using the iCyclerQ Sybr mix (Bio-Rad). Fluorescent PCR products were detected using an iCyclerQ real-time PCR detection system (Bio-Rad). Data were quantified using standard curves generated for each primer set and normalized to β-actin to control for total RNA concentration. Melting curves were generated for each sample after amplification for the determination of primer dimers, and product amplification was verified using acrylamide gel electrophoresis.

The primer sets are as follows: zf PEPCK forward 5'-GAGAATTCTCACACACAC ACACGTGAGCAGTA-3', reverse 5'-GTAAAAGCTTTCGCCATAACATCTCCAGC A GAA-3'; zf preproinsulin (*insa*) forward 5'-AGTGT AAGCACTAACCCAGGCACA-3', reverse 5'-TGCAAAGT CAGCCACCTCAGTTTC-3'; zf β-actin forward 5'-CGAG CAGGAGATGG GAACC-3', reverse- 5' CAACGGAAACG CTCATTGC-3' (Keegan *et al.* 2002).

Statistical analysis

Statistical analysis was performed using the SigmaStat v.1.0 computer software package (Systat Software, San Jose, CA, USA). The probability level for statistical significance was $P < 0.05$. Change in mRNA level was evaluated by one-way ANOVA and either Dunnett's test or Student–Newman–Keuls test was utilized as the multiple comparison method.

Results

Exposure of adult zebrafish to anti-diabetic compounds reduces blood glucose levels

There are various classes of anti-diabetic drugs, with different mechanisms of action (Fig. 1). Glipizide is a sulfonylurea, an insulin secretagogue that alters the bond equilibrium between insulin and β-cells of the pancreas and stimulates endogenous insulin release

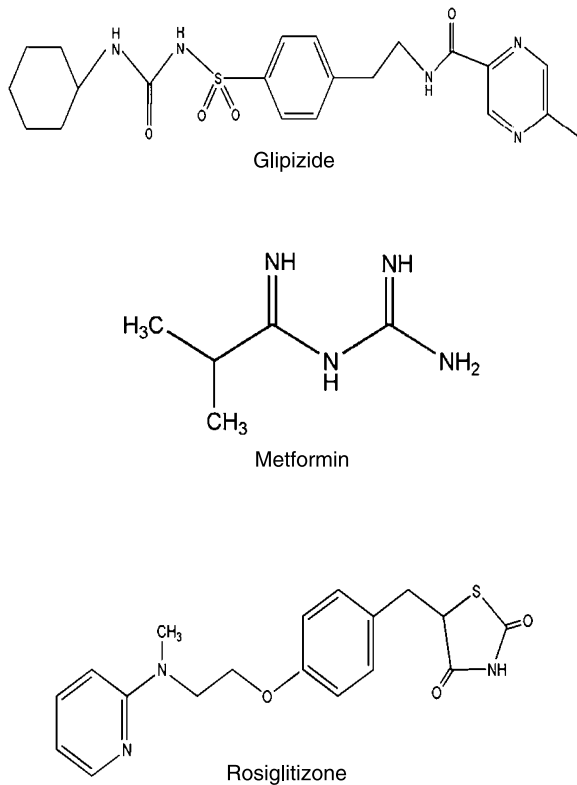


Figure 1 Structures of the anti-diabetic compounds used in the experiments.

(Rendell 2004). Metformin is a biguanide that reduces hepatic glucose production by facilitating glucose transporter activity into cells (Goodarzi & Bryer-Ash 2005). A third mechanism of anti-diabetic action is to sensitize the cell to insulin, such as the action of the thiazolidinediones (i.e. rosiglitazone (ROSI)), which bind to peroxisome proliferator-activated receptors and induces the transcription of metabolic enzymes (Pietruck *et al.* 2005).

Two methods of delivering glucose to adult zebrafish were used to determine the most efficient way to elevate blood glucose levels. Adult males were fasted for 48 h and either fed high-glucose flake food or transdermally exposed to glucose in Me_2SO by applying it to the abdomen of lightly anesthetized fish. Blood was collected from the zebrafish tail vein and glucose levels determined using a glucometer. Feeding of glucose did not significantly alter the blood glucose levels (Fig. 2A). However, transdermal absorption of the glucose effectively elevated the blood glucose. Therefore, all exposures in later experiments using the adult zebrafish were performed using transdermal exposure.

In order to determine whether known anti-diabetic drugs alter the zebrafish blood glucose levels, blood was

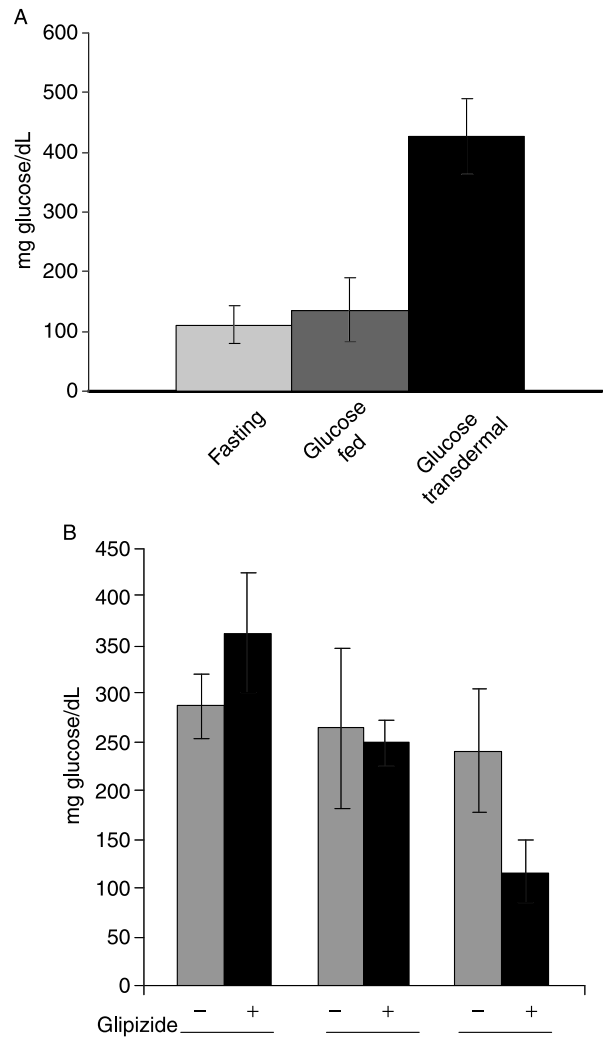


Figure 2 Effects on blood glucose levels in adult zebrafish. (A) Adult male zebrafish were fasted for 48 h and either fed glucose-rich flakes or exposed to a 25% glucose/ Me_2SO solution transdermally. Blood glucose was measured 45 min post-treatment using a OneTouch Ultra (Johnson & Johnson) blood glucometer. A minimum of six male zebrafish were used per treatment. (B) Adult male zebrafish were exposed to a 25% glucose/ Me_2SO solution transdermally with or without glipizide (1.5 mg/ml). Blood glucose levels were measured at 5, 15, and 24 h post-treatment.

collected from adult zebrafish that had been either loaded with glucose alone or co-treated with glucose and glipizide, an FDA-approved anti-diabetic drug. When zebrafish were co-exposed to glucose and glipizide, the blood glucose levels were significantly reduced in comparison with glucose alone by 24 h post-exposure (Fig. 2B). These data suggest that the regulation of blood glucose levels in zebrafish is similar to that observed in mammalian systems.

PEPCK expression is reduced by exposure of larval zebrafish to exogenous glucose

To develop the zebrafish as an appropriate model system for screening of compounds that alter glucose metabolism, we decided to use the larval zebrafish. Organ development, including the liver and pancreas, is completed by day 4 (96 hpf) of zebrafish development. Because larval zebrafish do not have the amount of blood necessary for accurate analysis of blood glucose levels, we opted to analyze the expression of PEPCK, which is transcriptionally regulated by glucagon and insulin and is therefore a sensitive marker of blood glucose levels (Yeagley *et al.* 2000, Cassuto *et al.* 2005, Chakravarty *et al.* 2005, Yeagley & Quinn 2005).

The expression of PEPCK in larval zebrafish was examined using real-time RT-PCR. Total RNA was isolated from larval zebrafish exposed to vehicle or glucose, and the expression of PEPCK was analyzed 24–48 h post-treatment. The data show that expression of PEPCK was detectable in larval zebrafish, and that exogenous glucose significantly inhibited its expression at both 24 and 48 h post-exposure (Fig. 3A). These data indicate that PEPCK is a sensitive marker for blood glucose levels in larval zebrafish.

The expression of zebrafish preproinsulin was also examined following exposure to exogenous glucose. Although there are two zebrafish insulin genes, *insa* and *insb*, the expression of *insb* appears to be restricted to early times in embryonic development (Papasani *et al.* 2004). Therefore, we focused on the expression of *insa*, which is expressed in the larval zebrafish. The data show that preproinsulin expression was upregulated by exposure to glucose at both the 24- and 48-h time points (Fig. 3B). These data indicate that the larval zebrafish is absorbing the exogenous glucose and is responding to the increased blood glucose levels as expected.

PEPCK expression is induced by cAMP + dexamethasone and retinoic acid

PEPCK is primarily regulated at the level of transcription. Glucagon activates the expression of PEPCK through the activation of cAMP leading to increased binding to cAMP elements in the PEPCK promoter (Leahy *et al.* 1999, Yamada *et al.* 1999, Herzig *et al.* 2001, Liu *et al.* 2001, Monroy *et al.* 2001, Waltner-Law *et al.* 2003). In addition, glucocorticoids, atRA, and thyroid hormone activate *PEPCK* gene expression (Stafford *et al.* 2001, Waltner-Law *et al.* 2003). Therefore, to demonstrate activation of PEPCK expression in the larval zebrafish, we exposed the embryos to cAMP and the glucocorticoid DEX (Fig. 4A). Exposure of zebrafish to cAMP and DEX increased PEPCK mRNA levels 48 h post-exposure. DEX increased PEPCK in a dose-dependent manner. Next,

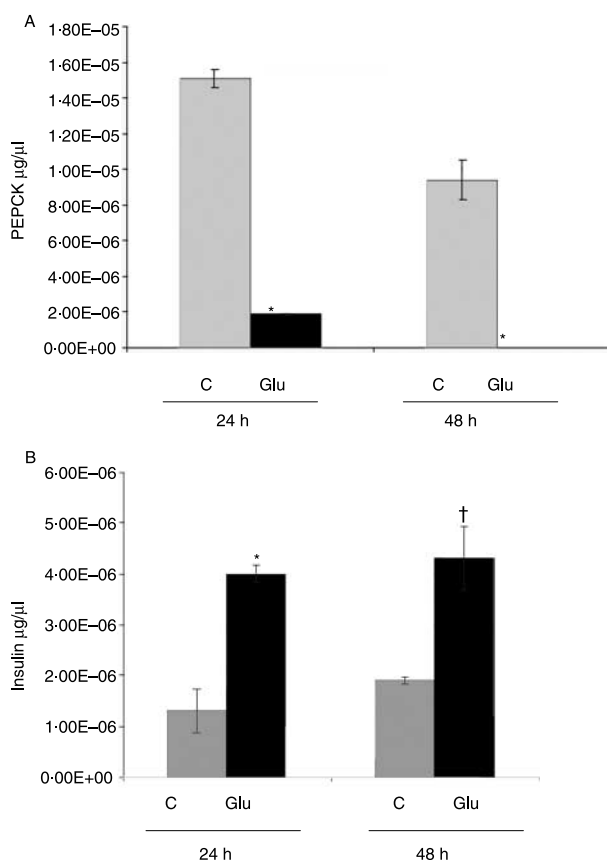


Figure 3 Expression of PEPCK is reduced following exposure of larval (96 hpf) zebrafish. Total RNA isolated from larval zebrafish exposed to 40 mM glucose (GLU) for 24 and 48 h was used as a template for quantitative RT-PCR using primers specific for zfPEPCK (A), zf-preproinsulin (B), and β -actin. PCR products were detected using SYBR green as a fluorogenic marker and an icyclerq (Bio-Rad). Data were quantified using a standard curve generated using specific PCR products at concentrations ranging from 100 ng to 1 pg. Data were normalized to β -actin to control for total RNA concentration. Data were plotted as concentration of template after normalization to total RNA concentration by β -actin. Error bars denote s.d. Statistical significance between control and untreated expression, determined by Student's *t*-test, is denoted by * $P \leq 0.005$ or † $P \leq 0.02$.

larval zebrafish were exposed to atRA for 48 h, and PEPCK expression was determined using RT-PCR. Data show that exposure to atRA results in a twofold increase in the PEPCK expression (Fig. 4B). These data demonstrate that activation of PEPCK expression in larval zebrafish is regulated similarly to PEPCK in mammalian models.

Inhibition of PEPCK expression by known anti-diabetic compounds

To investigate the effect of anti-diabetic drugs, larval zebrafish were exposed to cAMP+DEX to elevate

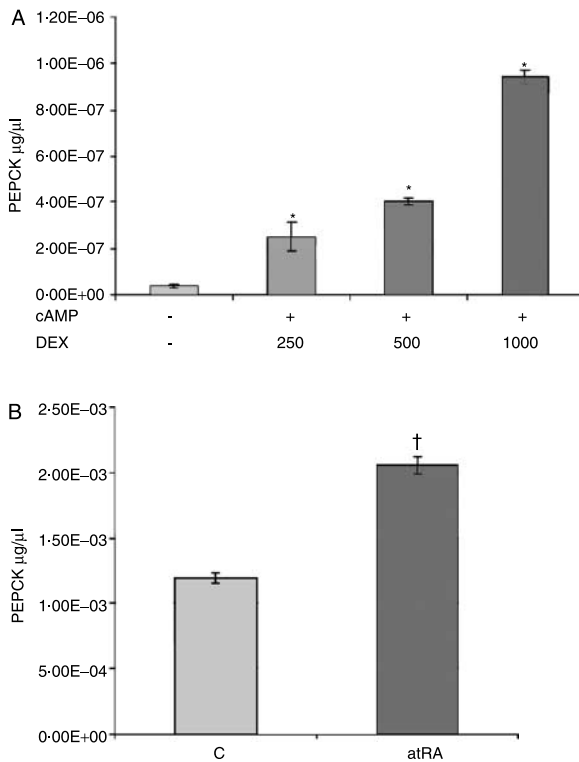


Figure 4 PEPCK expression is enhanced following exposure to cAMP+DEX or atRA. (A) Total RNA isolated from larval zebrafish exposed to cAMP (100 µM) and DEX (250, 500, and 1000 nM) for 48 h was used as a template for quantitative RT-PCR using primers specific for zfPEPCK and β-actin as described in Fig. 3. Error bars denote s.d. Statistical significance, determined by Student's *t*-test, is denoted by **P*<0.005. (B) Total RNA isolated from larval zebrafish exposed to atRA (10 nM) for 48 h was used as a template for quantitative RT-PCR as described in (A). Statistical significance, as determined by Student's *t*-test, is denoted by †*P*<0.001.

PEPCK mRNA levels, and co-treated with either MET, GLIP, or ROSI. Co-treatment with metformin resulted in an inhibition of cAMP+DEX activation of PEPCK expression by approximately 75% (Fig. 5B). Similarly, exposure to glipizide reduced PEPCK expression by 75–80% (Fig. 5A).

The final anti-diabetic compound tested, rosiglitazone, functions by enhancing insulin sensitivity of extrahepatic tissues (Pietruck *et al.* 2005). Our data show that exposure of larval zebrafish to rosiglitazone alone results in a slight but significant decrease in PEPCK expression (Fig. 5C). Furthermore, co-exposure with rosiglitazone and cAMP/DEX results in a slight but significant decrease in cAMP+DEX-induced PEPCK expression (Fig. 5C). Taken together, these data indicate that PEPCK expression is a sensitive indicator of blood glucose levels in larval zebrafish and demonstrate that PEPCK regulation in zebrafish is similar to that seen in humans and mice.

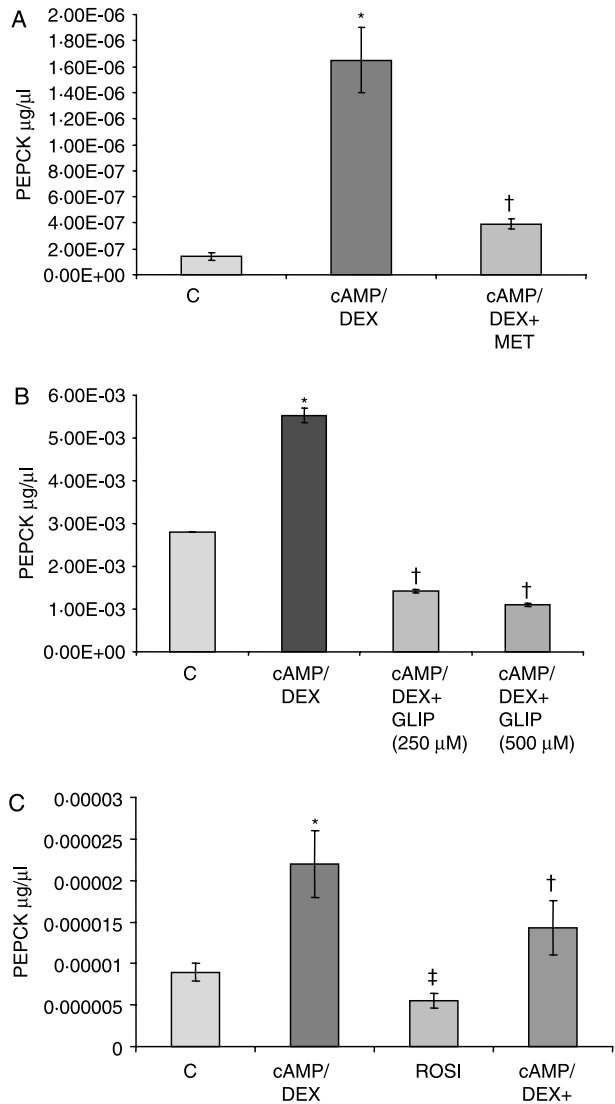


Figure 5 Anti-diabetic compounds reduce PEPCK expression in larval zebrafish. Total RNA isolated from larval zebrafish (96 hpf) exposed to cAMP+DEX or cAMP+DEX and (A) metformin (MET: 10 µM), (B) glipizide (GLIP: 250 or 100 µM), (C) cAMP+DEX+rosiglitazone (1 µM) or rosiglitazone (ROSI) alone for 48 h and was used as a template for quantitative RT-PCR using primers specific for zfPEPCK and β-actin as described in Fig. 3. Error bars denote s.d. Statistical significance, determined by Student's *t*-test, is denoted by **P*<0.001; †*P*≤0.005; ‡*P*<0.0001.

Discussion

The zebrafish is rapidly becoming one of the most widely used animal models for pharmacological and toxicological testing of new compounds. The data presented in this paper indicate that both the adult and larval zebrafish are appropriate models to test the effects of compounds on glucose mobility and

metabolism. Adult zebrafish blood samples demonstrated that the FDA-approved anti-diabetic drug, glipizide, lowers blood glucose levels in this model system. Data also demonstrate that these compounds act similarly in the larval zebrafish, through examination of PEPCK expression.

The larval zebrafish (96 hpf) is ideal for these studies, since hundreds of embryos can be analyzed simultaneously and organ development has been completed. The zebrafish pancreas, like that of mammalian species, results from the fusion of two buds (ventral anterior and dorsal posterior) emerging from the gut tube at the contact point of the endoderm and the vasculature (Field *et al.* 2003). The posterior bud gives rise to the endocrine portion of the tissue, whereas the anterior bud provides the exocrine function. As early as 34 hpf, the posterior bud is present as a cluster of cells that are already expressing endocrine specific genes. By 52 hpf the anterior and posterior buds have fused, with the anterior bud connecting to the alimentary canal and the posterior bud forming the islet. At 76 hpf, the exocrine pancreatic tail is observed, and the islet is completely engulfed by the exocrine tissue. Specific genes are critical for the proper development of the pancreas and are conserved between mammalian and zebrafish model systems. These include one of the earliest markers of pancreatic development, the pancreatic and duodenal homeobox-1 (*pdx-1*) gene (Milewski *et al.* 1998). Knockdown of *pdx-1* in zebrafish results in a reduction of both endocrine and exocrine tissues, with reduced numbers of exocrine cell types, as well as insulin-expressing endocrine cells. Another pathway of interest is the hedgehog signaling pathway (Hh). Sonic-youuo (*syu*)/sonic hh (*shh*) mutant embryos have reduced expression of *pdx-1*, in addition to reduced levels of the endocrine-specific markers, insulin and glucagon (Roy *et al.* 2001). However, no apparent reduction in exocrine-specific gene expression is observed, indicating that the *shh* pathway is specific for the development of the endocrine tissue. These data indicate that pancreatic development in zebrafish is regulated by similar pathways as in mammalian model systems.

Two insulin genes have been identified in zebrafish (*insa* and *insb*; Papanasi *et al.* 2004, Fostel *et al.* 2005). This is hypothesized to be the result of a genome duplication event that occurred prior to the divergence of teleost fish. In most cases of gene duplication, the duplicated gene is thought to become a non-functional pseudogene because there is no selective advantage to maintain them (Force *et al.* 1999). However, in some cases, the duplicated genes are functionally maintained if they either acquire a new function or the duplicated gene maintains only certain specialized functions of the primary gene. In the case of the two insulin genes in zebrafish, both genes have maintained functionality; however, their expression

patterns differ. Both *insa* and *insb* contain the proteolytic processing sites to convert the proinsulin to insulin (Irwin 2004), and these data indicate that the insulins are processed similarly to mammalian insulin and that the processed zebrafish insulins have a three-dimensional structure that is similar to the mammalian protein (Irwin 2004). By analysis of developmental expression patterns, it is believed that *insa* functions much like the mammalian homologue in glucose regulation, whereas the *insb* is believed to play a more important role in development (Papanasi *et al.* 2004).

To investigate the effects of select anti-diabetic compounds in larval zebrafish, we chose to examine the expression of PEPCK. PEPCK catalyzes the first committed step in gluconeogenesis, the conversion of oxaloacetate to phosphoenolpyruvate. Although acute hyperglycemia suppresses the expression of genes involved in gluconeogenesis, including PEPCK, chronic hyperglycemia is associated with increased hepatic glucose production and increased PEPCK expression (Quinn & Yeagley 2005). The ability of a compound to alter PEPCK expression has been used to determine the efficacy of anti-diabetic compounds in mammalian cell culture models. Furthermore, chronic hyperglycemia, like that observed in type II diabetes, results in increased PEPCK expression and activation of gluconeogenesis. The ability of compounds to downregulate PEPCK is an important aspect of their function as anti-diabetic drugs.

The mammalian PEPCK promoter region contains cis-acting elements that mediate activation by glucocorticoids, glucagon, thyroid hormone, and retinoic acid (Quinn & Granner 1990, Hall *et al.* 2000, Monroy *et al.* 2001, Waltner-Law *et al.* 2003). It has been determined that the proximal promoter (−477 to −1 bp) contains the sequences required for positive regulation of PEPCK, and that this region also contains sequences that are necessary for glucose-mediated inhibition (Cournarie *et al.* 1999). Additional elements upstream contribute to the magnitude of the transcriptional activation by glucocorticoids; however, they are not necessary for activation. Work from some laboratories indicate that insulin inhibition of PEPCK does not appear to require specific insulin responsive elements, but instead works by inhibiting activation of the gene through blocking binding to the activation elements. However, a PEPCK-like motif in the promoter is implicated in insulin inhibition (O'Brien *et al.* 2001).

The entire zebrafish genome has been sequenced and is available online (National Center for Biotechnology Information (NCBI)). The zebrafish *PEPCK* gene is located on chromosome 17, and approximately 1.2 kb of the 5' regulatory sequence is available. Analysis of the potential zfPEPCK promoter using a transcription factor database (MatInspector) revealed several elements that are also found in the

human promoter, including the cAMP-binding sites and CAAT enhancer, which mediate activation by glucagon, and several AP-1 elements and serum response elements, as well as elements involved in tissue and developmental specific regulation (PDX, PAX; Ghosh 1991, 1992). The zebrafish PEPCK promoter also contains several hepatic nuclear factor 3 β elements, and several PEPCK-like motifs are also present in this 1.2 kb sequence.

The similarity between the mammalian and zebrafish PEPCK promoters suggests that the zPEPCK promoter contains the regulatory elements necessary for proper tissue-specific expression, as well as expression in response to glucagons, glucocorticoids, and insulin. The data presented in this paper confirm the similarity in the regulation of the zebrafish and mammalian PEPCK genes. The cAMP and the glucocorticoid dexamethasone activate PEPCK expression, as does exposure to atRA. Furthermore, acute hyperglycemia caused a significant reduction in PEPCK expression.

The anti-diabetic compounds used in this study all regulate blood glucose levels through distinct mechanisms. Glipizide and related sulfonylureas stimulate insulin release, in addition to affecting glucose and insulin sensitivity in extrahepatic tissues (Rendell 2004). Metformin inhibits the endogenous production and release of non-carbohydrate-derived glucose (Goodarzi & Bryer-Ash 2005). Rosiglitazone is a thiazolidinedione, which binds to the peroxisome proliferator-activated receptors (PPARs), and functions as an anti-diabetic compound by enhancing the sensitivity of the peripheral tissues to insulin (Pietruck *et al.* 2005). Our data also show that the FDA-approved anti-diabetic drugs metformin, glipizide, and rosiglitazone reduce the expression of zPEPCK. Given that chronic hyperglycemia activates PEPCK expression and ultimately gluconeogenesis, one critical function of these compounds is to reduce PEPCK expression. Our data in zebrafish is similar to that seen in mammalian tissue culture. For example, sulfonylurea and metformin exposure reduce PEPCK expression in liver cell lines and rat hepatocytes respectively (Davies *et al.* 1999, Yuan *et al.* 2002). Rosiglitazone can directly affect PEPCK expression, via PPAR elements in the promoter region (Duplus *et al.* 2003). However, data from hepatocytes indicate that rosiglitazone is not effective in regulating the PEPCK expression in this model (Davies *et al.* 2001), causing only a slight decrease. This is similar to our data showing that in the larval zebrafish, exposure to rosiglitazone results in a slight but significant decrease in PEPCK expression.

In summary, our data demonstrate that regulation of PEPCK in larval zebrafish mimics that seen in mammalian cells. This is further supported by the data from other laboratories demonstrating that the ortholog to the human glucose transporter GLUT1 has

been identified in zebrafish (Jensen *et al.* 2006). These findings suggest that the larval zebrafish may be an ideal model in which to examine the effects of novel compounds on glucose metabolism and transport.

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