Nutritionally induced insulin resistance in an Indian perch: a possible model for type 2 diabetes

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We have developed nutritionally induced, insulin-resistant Indian perch, *Anabas testudineus*, a freshwater teleost which showed all the characteristics found in type 2 diabetes mellitus. Free fatty acids (FFAs) are known to be the major players in implementing insulin resistance and type 2 diabetes mellitus, an epidemic disease threatening global health. Prolong feeding of a FFA, palmitic acid, to perch induced hyperinsulinaemia, hyperglycaemia, loss of insulin sensitivity and increase in glycosylated haemoglobin (HbA_{1C}). Skeletal muscle cells from FFA-treated perch showed defects in insulin signalling molecules suggesting its link to the impairment of insulin action, a situation similar to that found in insulin-resistant mammalian species and human beings. Results indicate that this nutritionally induced insulin-resistant perch could be a convenient animal model to examine the potential of therapeutic compounds, addressing the problems of insulin resistance and type 2 diabetes.

Keywords: Diabetes model, glucose transport, insulin resistance, insulin signalling, type 2 diabetes mellitus.

TYPE 2 diabetes or non-insulin-dependent diabetes mellitus (NIDDM) is an epidemic disease seriously threatening global human health. In type 2 diabetes, insulin level is normal or more, but its action on target cells is attenuated due to defects in signal transduction pathway, which restricts glucose uptake by target cells. In type 1 diabetes or insulin-dependent diabetes mellitus (IDDM), insulin level is below normal due to pancreatic β-cell damage. More than 95% diabetic patients are type 2 diabetic and the prevalence of this disease has rapidly increased recently. Type 2 diabetes therefore requires serious research attention with the pressing need for animal models to pursue the development of new therapeutic agents. Varieties of animal models such as rats[1], dogs[2], Chinese hamsters[3], swine[4], non-human primates[5] and other endothermic vertebrates representing both type 1 and type 2 diabetes have been developed till date. However, attempts to develop diabetes mellitus models in teleostean fish and in ectothermic vertebrates in general have met with difficulties. The drug streptozotocin, for instance, has no diabetogenic effect in teleosts, whereas alloxan may sometimes cause diabetic symptoms often associated with a significant degree of systemic toxicity[6]. Alternatively, a surgical approach to selectively remove pancreatic endocrine organs called principal islets or Brockmann bodies to develop an insulin-deficient model has been successful in the lamprey, *Geotria australis*[7] and the goby, *Gillichthys mirabilis*[8], but neither of these models could address type 2 diabetes where hyperglycaemia persists with normal or higher levels of circulatory insulin.

Nutritionally induced type 2 diabetes in *Psammomys obessus* or sand rat has been well documented[9–12]. *P. obessus* develops hyperinsulinaemia and hyperglycaemia when transferred to a relatively high-calorie diet[9]. Intake of high calorie diet and increased level of free fatty acids (FFAs) has been shown to have a relationship in the development of type 2 diabetes. Elevated FFAs in circulation is associated with impaired insulin function and is commonly linked with obesity and type 2 diabetes[13–15]. Rising of plasma concentration through lipid infusion causes insulin resistance in rat and human skeletal muscle[15–17]. A number of reports also showed palmitate as the most potent FFA in inhibiting insulin action or causing insulin resistance[18–21]. Taking all the information into account, we initiated an investigation on an Indian perch, *Anabas testudineus* Bloch, to induce insulin resistance through prolonged feeding of FFA. *Anabas* is an unusual freshwater fish, having accessory respiratory organ to breathe air along with the gills required for aquatic life. It is a small-sized fish and can crawl and climb because of its air-breathing habit. All these attributed toughness and therefore an excellent biological speciman for laboratory experiments. Nutritionally induced insulin-resistant perch could be a good model to study type 2 diabetes mellitus, as they develop hyperinsulinaemia and hyperglycaemia. We have also demonstrated that FFA-induced development of insulin-resistant perch is due to insulin signalling defects in the skeletal muscle cells, a major characteristic of type 2 diabetes.

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Materials and methods

Reagents

All tissue culture materials were obtained from Gibco-BRL, Life Technologies Inc., Gaithersburg, MD, USA. Palmitate, trypsin, porcine insulin and calfskin collagen (type III) were purchased from Sigma Chemical Company, St Louis, MO, USA. 2-Deoxy-[1-^{3}H] glucose (specific activity: 12.0 Ci/mmol) was from Amersham Biosciences, USA. Antibodies utilized included anti IR-β (anti-rabbit), anti-p-Tyr (anti-mouse) anti-p-IRS 1 (anti-goat), anti-p-PI3K (anti-goat), anti-p-Akt 1/2/3 (anti-rabbit) from Santa Cruz Biotechnology, Inc, USA. Anti-p-PDK1 (anti rabbit) antibody was from Cell Signaling Technology, Inc, USA. Alkaline phosphatase-conjugated anti-rabbit and anti-goat secondary antibodies were also purchased from Santa Cruz Biotechnology Inc., USA. All other chemicals were from Sigma.

Animal experiments

Adult *Anabas* were collected from in and around Santiniketan, West Bengal, India and were maintained under controlled laboratory conditions in a cemented water tank, which was well aerated with both inlet and outlet pipes. Initially all the fish were allowed to acclimatize for two days. Perch having approximately equal size (9.0–11.0 cm) and body weight (35.0–39.0 g) were selected for experiments and collected during preparatory stage of their annual reproductive cycle. During the experiments special floating-type pelleted feed were provided which contained Spirulina along with essential proteins. The fish were separated into two groups (control and palmitate-fed) and their initial body weights were recorded before experimentation. Both the groups availed pelleted feed *ad libitum*; the treated group was additionally fed with palmitate (150 μg/100 g body wt daily) by inserting a feeding tube through the mouth, fitted with a syringe. This was carried out according to the technique used in other animals to administer specific amount of experimental feed. We used various amounts of palmitate from 50 to 300 μg/100 g body wt and found 150 μg/100 g body wt to be the minimum dose to produce hyperinsulinaemia and hyperglycaemia on day-100. For the experiment, 100-day FFA-fed perch were used with clear manifestation of hyperglycaemia and hyperinsulinaemia.

Estimation of plasma glucose and insulin

Blood was drawn from the caudal vein of the fish with the help of a heparinized 2 ml disposable syringe and immediately centrifuged to collect the plasma. Blood glucose was determined by the glucose–glucose oxidase method (glucose test kit, Span Diagnostic, India). Plasma insulin was estimated with the help of insulin RIA. Fish insulin RIA was developed in our laboratory with the collaboration of Katsumi Wakabayashi, Institute for Molecular and Cellular Regulation, Gunma University, Japan. We examined both anti-porcine insulin antibody (from the laboratory of Wakabayashi) and anti-salmon insulin antibody (from Erika Plisetskaya, University of Washington, Seattle, USA) and found similar cross-reaction with our carp and perch insulin. However, we have used anti-porcine insulin because of its regular availability. This RIA was also used in our earlier study with carp insulin[22].

Incubation of perch skeletal muscle cells

White muscle fibres isolated from rostral myotomes designated to be glycolytic and highly active, were dissected out from perch and are referred to here as perch skeletal muscle. The skeletal muscle tissue from control and palmitate-fed perch was thoroughly washed with the culture medium and subjected to digestion with trypsin (0.02%) and collagenase (0.5%) for 30 min at 35 ± 2°C in DMEM (Dulbecco's Modified Eagle's medium) in the presence of 95% O_2 and 5% CO_2. The dispersed skeletal muscle cells were pelleted by centrifugation at 500 *g*, washed and resuspended in DMEM containing 10% foetal calf serum and 1% penicillin/streptomycin. Control and palmitate-fed perch skeletal muscle cells were incubated with 100 nM insulin for 30 min followed by determination of [^{3}H] 2-deoxy-glucose (2-DOG) or Western blot analysis using phospho-specific antibodies.

[^{3}H] Deoxyglucose uptake

[^{3}H] Deoxyglucose (Amersham Biosciences, USA) uptake by the skeletal muscle cells from perch was conducted by following a previously described procedure from our laboratory[22]. Briefly, cells were serum-starved overnight in Kreb's Ringer Phosphate (KRP) buffer (12.5 mM HEPES, pH 7.4; 120 mM NaCl; 6 mM KCl; 1.2 mM $MgSO_4$; 1 mM $CaCl_2$; 0.4 mM NaH_2PO_4; 0.6 mM Na_2HPO_4) supplemented with 0.2% bovine serum albumin. Skeletal muscle cells from control and palmitate-fed perch were incubated with porcine insulin (100 nM) for 30 min [^{3}H] 2-DOG (0.4 nmol/ml) was added 5 min prior to the termination of incubation. Cells were washed thrice with ice-cold KRP buffer in the presence of 0.3 mM phloretin to correct the [^{3}H] 2-DOG uptake data from simple diffusion and non-specific trapping of radioactivity. Cells were then harvested with trypsin (0.25%)-EDTA (0.5 mM), solubilized with 1% NP-40 and [^{3}H] 2-DOG count was determined in a liquid scintillation counter (Packard, Tricarb 2100 TR).

Electrophoresis and immunoblotting

Perch skeletal muscle cell pellets obtained after washing as described above were resuspended in lysis buffer (1%

NP-40, 20 mM HEPES (pH 7.4), 2 mM EDTA, 100 mM NaF, 10 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 1 μg/ml pepstatin and 1 mM PMSF) and sonicated on ice at 150 kHz for 5 min. Lysates were centrifuged for 10 min at 10,000 *g*, supernatant was collected and protein content estimated following the method described by Lowry *et al.* Control and treated cell lysates (50 μg) were resolved on 10% SDS–PAGE and transferred to PVDF membranes (Millipore, Bedford, MA, USA) through transfer buffer (25 mM Tris, 193 mM glycine, 20% methanol; pH 8.5) for 1.5 h at 4°C at 90 V. Membranes were blocked with 5% non-fat dried milk in TBST buffer (20 mM Tris base, 137 mM NaCl, 1 mM HCl, 0.1% Tween 20) and incubated overnight with phospho-specific antibodies such as anti p-IRS 1 (anti-goat; 1 : 1000), anti p-PI3K (anti-goat; 1 : 1000), anti p-Akt 1/2/3 (anti-rabbit; 1 : 1000) and anti p-PDK1 (anti-rabbit 1 : 1000) antibodies. A concurrently run gel was immunoblotted with anti IRS 1 (anti-goat; 1 : 1000), anti PI3K (anti-goat; 1 : 1000) and anti Akt 1/2 (anti-rabbit; 1 : 1000) antibodies. Immunoreactive bands were detected with alkaline phosphatase-linked secondary antibodies.

Immunoprecipitation

To observe insulin receptor tyrosine kinase phosphorylation, control and palmitate-fed skeletal muscle cells were incubated with porcine insulin (100 nM) for 30 min, lysed by sonication in lysis buffer (as described above) followed by centrifugation at 10,000 *g* for 10 min. Supernatant was collected and 200 μg of protein was incubated overnight at 4°C with 2 μg insulin receptor-β (IR-β) antibody. Next 50 μl of protein A-agarose was added to each tube and incubated at 4°C for 2 h followed by centrifugation at 10,000 *g*. The pellet was resuspended in 500 μl of 0.1% CHAPS in PBS, washed thoroughly and subjected to SDS–PAGE followed by immunoblotting with anti-phosphotyrosine antibody (anti-mouse; 1 : 1000).

Statistical analysis

Data were analysed by one-way analysis of variance (ANOVA), where the *F* value indicated significance, means were compared by a post hoc multiple range test. All values were means ± SEm.

Results

FFA-induced hyperinsulinaemia and hyperglycaemia in perch

Palmitate intake for a prolonged period (100 days) increased the body weight to more than 60% in comparison to control. Both palmitate and control group received the same food pellet *ad libitum*. Initial body weight of experimental perch was 35.0 ± 3 g; after 100 days of feeding control perch gained about 6 g, while palmitate-fed perch gained approximately 25 g. Palmitate feeding in addition to normal food affected unexpected increase in body weight. Plasma glucose and insulin levels in palmitate fed group were elevated to more than 2.5 fold on 100 day compared with the control. There was a significant increase ($P < 001$) in glycosylated haemoglobin (HbA_{1C}) level in FFA-fed perch compared to control (Table 1). These results indicate that FFA, i.e. palmitate feeding caused insulin resistance and pathogenicity similar to type 2 diabetes.

Development of insulin resistance

One of the dependable assays to determine insulin resistance and type 2 diabetes is to examine uptake of glucose by skeletal muscle cells from hyperinsulinaemic animals; inability of insulin to stimulate glucose uptake suggests development of insulin resistance. We have taken skeletal muscle cells from both control and 100 days palmitate-fed perch which developed hyperinsulinaemia, incubated without (control) or with insulin in the presence of [^{3}H] 2-DOG. Figure 1 *a* shows that insulin markedly increased [^{3}H] 2-DOG uptake in skeletal muscle cells from control perch, while this was significantly inhibited ($P < 0.001$) in skeletal muscle cells of FFA-fed group. This indicates an impairment of insulin activity due to FFA; loss of insulin sensitivity is an indication of insulin resistance or type 2 diabetes. [^{3}H] 2-DOG uptake in skeletal muscle cells from FFA-treated perch was marginally reduced compared to control. Although this was statistically insignificant, it suggests imposition of a defect in the skeletal muscle. Impairment of insulin sensitivity is usually linked to inhibition of insulin-stimulated signals. We selected phosphorylation of IR-β, since it is the first upstream signal as a consequence of insulin binding to the α-domain of IR. Insulin-stimulated IR-β tyrosine phosphorylation was clearly reduced by palmitate (Figure 1 *b*).

Inhibition of insulin-mediated signalling molecules

Reduction of insulin stimulated [^{3}H] 2-DOG uptake and inhibition of IR tyrosine kinase phosphorylation in the skeletal muscle cells indicates damage in the insulin signalling cascade. We therefore examined insulin-augmented downstream signals, which get phosphorylated following IR tyrosine kinase phosphorylation. Using phospho-specific antibodies, we observed that insulin stimulation of IRS1 (Figure 2 *a*) and PI3 kinase (Figure 2 *b*) phosphorylation in skeletal muscle cells were inhibited due to palmitate intake. PI3 kinase converts phosphoinositides to phosphotidylinositol 3,4,5-triphosphate that recruits PDK-1; which promotes Akt/PKB activation by facilitating phosphorylation of its regulatory domains. Activated Akt promotes

Table 1. Hyperinsulinaemia and hyperglycaemia in palmitate-fed perch. Both control and palmitate-fed perch received pelleted food *ad libitum*. In the treated group, each perch was additionally fed with 150 µg palmitate/100 g body wt daily. Procedure for palmitate feeding is described in the text. Body weight of each fish was recorded on day 100, blood was collected and subjected to glucose, insulin and HbA_{1C} estimation. Values expressed are ± SEm of five independent determinations taking seven fish for control or palmitate-fed group for each observation. *$P < 0.01$, **$P < 0.001$ when compared with control

	Body wt (g)		Glucose(mg/dl)		Insulin (ng/ml)		% HbA_{1C}	
System	0 day	100 days	0 day	100 days	0 day	100 days	0 day	100 days
Control	35.0 ± 3.0	41.0 ± 2.0	100.0 ± 5.0	101.0 ± 7.0	5.5 ± 0.7	5.7 ± 1.0	2.1 ± 0.2	2.5 ± 0.1
Palmitate-fed	37.0 ± 2.0	62.0 ± 5.0*	110.0 ± 7.0	276.5 ± 6.0**	5.9 ± 0.5	17.28 ± 2.3**	2.7 ± 0.3	9.5 ± 1.0**

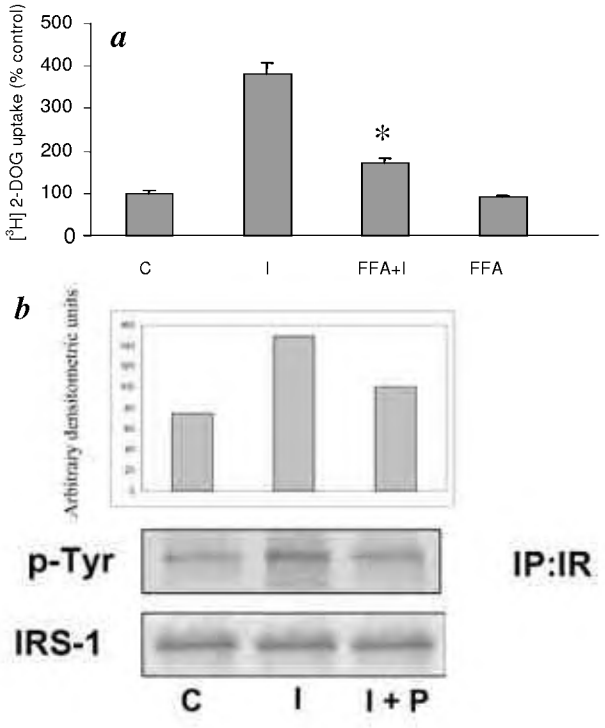

Figure 1*a*. Effect of FFA on [^{3}H] 2-DOG uptake in perch skeletal muscle cells. Skeletal muscle cells were isolated from both control and FFA, i.e. palmitate-fed groups and incubated with 100 nM insulin for 30 min. [^{3}H] 2-DOG was measured in the final 5 min of treatment as described in the text. Results depict mean ± SEm of four independent experiments compared to incubation with insulin alone ($n = 5$ for each experiment). C, Muscle cells from normally fed perch; I, Muscle cells from control perch incubated with insulin; FFA, Skeletal muscle cells from palmitate-fed perch; FFA + I, Skeletal muscle cells from palmitate-fed perch incubated with insulin. *Significance at $P < 0.001$ compared to insulin effect. ***b***, Inhibition of insulin-stimulated insulin receptor tyrosine kinase activation by palmitate in skeletal muscle cells. Skeletal muscle cells from control and palmitate-fed perch ($n = 3$ for each set) were isolated by trypsin-collagenase digestion, treated with insulin (100 nM) for 30 min, immunoprecipitated with 2 µg IR-β antibody followed by immunoblotting with anti-p-Tyr antibody. C, Skeletal muscle cells from control perch; I, Control muscle cells incubated with insulin; P, Skeletal muscle cells from palmitate-fed perch incubated with insulin. IRS-1 was used as a loading control.

translocation of glucose transporter Glut4 to the cell membrane to bring glucose into the cell. Palmitate disrupts insulin-stimulated phosphorylation of both these downstream molecules, i.e. PDK1 and Akt/PKB (Figure 2*c*) and therefore opposes insulin signalling activity causing insulin resistance. In palmitate-treated perch, phosphorylation of PDK-1 and Akt/PKB was slightly reduced compared to control.

Reversibility of FFA-induced insulin resistance

Perch fed for 100 days were allowed withdrawal of palmitate for 25 days, and an analysis of plasma level glucose, insulin and HbA_{1C} demonstrated a significant decline in their values compared to the palmitate-fed group (Figure 3). This indicates reversibility of FFA-induced insulin resistance in perch.

Discussion

Nutritionally induced insulin-resistant perch showed similar characteristics as observed in type 2 diabetic patients[23–25]. Continuous feeding of FFA to perch promoted development of hyperinsulinaemia and hyperglycaemia along with significantly high levels of glycosylated haemoglobin (HbA_{1C}). These suggest that nutritionally induced insulin-resistant perch can be conveniently used as a model for type 2 diabetes mellitus. Till date only mammalian species are available as a model for type 2 diabetes. However, there are attempts to develop models for type 1 diabetes with different species of teleostean fish with varied degrees of success[7,26,27]. We have selected this perch species due to several advantages: (a) it can be easily maintained under laboratory conditions without mortality because of its air-breathing ability, (b) it can be bred under laboratory conditions and (c) it can bear surgery stress and recuperate quickly after surgery. All these indicate the perch to be a convenient specimen for laboratory use.

Since skeletal muscle is the major target tissue of insulin due to its maximum glucose storage ability (about 75%), it is most suitable for the study of pathogenesis due to type 2 diabetes. Recent studies suggest that increased circulatory FFA levels and elevated intramuscular lipid concentrations have a strong relationship with insulin resistance and type 2 diabetes[16,17,28,29]. Some reports have also demonstrated that FFAs induce insulin-resistance by inhibiting insulin-stimulated phosphorylation of IRS1 and IRS1-

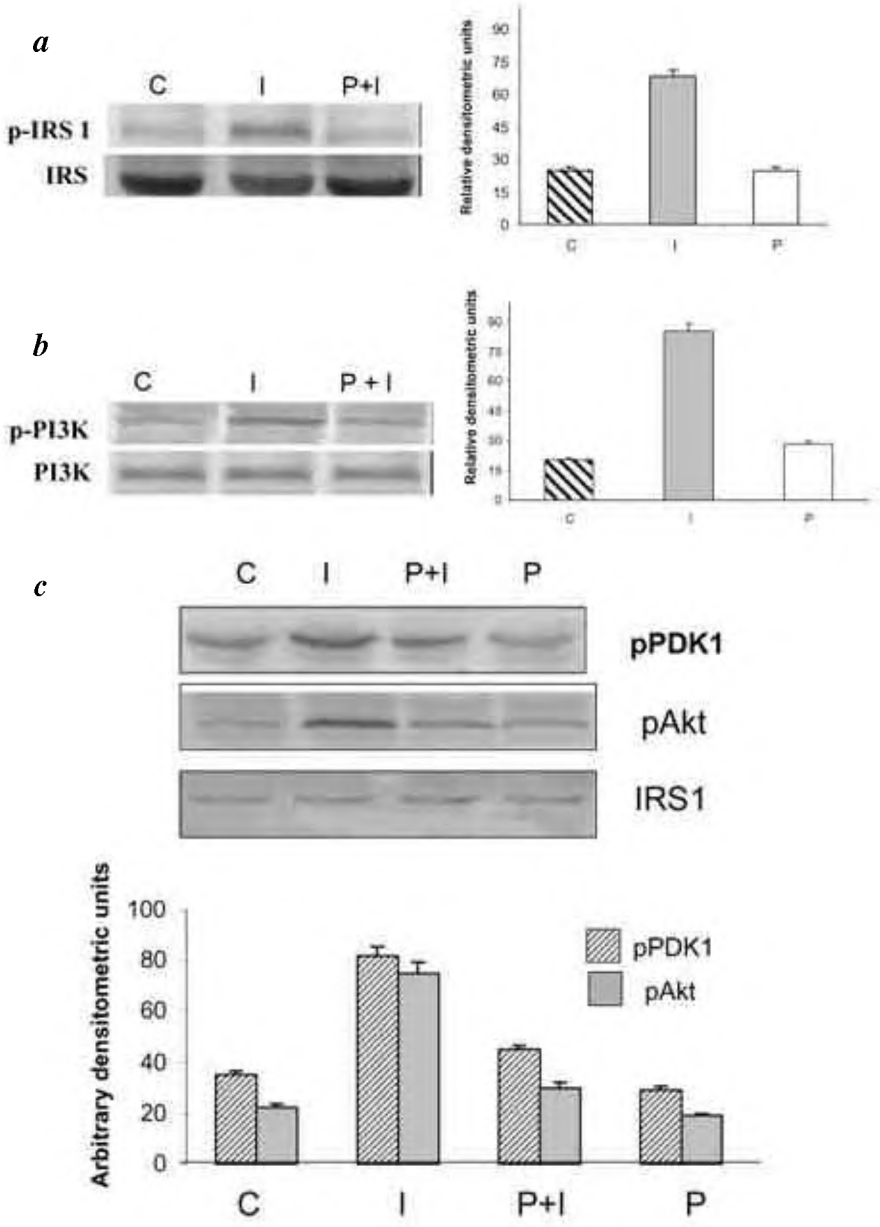

Figure 2. Inhibition of insulin signalling molecules by palmitate. Skeletal muscle cells from control and palmitate-fed groups (for each immunoblot, three fishes from treated and control groups were taken; total treated = 9, total control = 9) were incubated with insulin as described in the text. On termination of incubation, cells were lysed by sonication in lysis buffer and centrifuged at 10,000 *g* for 10 min. Supernatant protein (50 μg each) was boiled in SDS–PAGE sample buffer and resolved on 10% SDS–PAGE followed by transfer to PVDF membrane and immunoblotted with anti-p-IRS-1, or anti-p-PI3 kinase p85α or anti pPDK-1 and anti-p-Akt-antibodies. Anti-protein antibodies of IRS1 and PI3 kinase p85α were used as loading control. All antibodies were used in 1 : 1000 dilution. C, Control; I, Control plus insulin, P, Palmitate; P + I, Palmitate plus insulin.

associated PI3 kinase[30,31]. Sand rat (*P. obessus*) is a good model for nutritionally induced type 2 diabetes, where regular intake of high energy diet promoted the development of hyperinsulinaemia and hyperglycaemia[11,12]. In perch, a fairly long time is required to develop hyperglycaemia and hyperinsulinaemia. A significant increase in blood glucose and insulin level in FFA-fed perch was observed on day 100, which maintained a steady state till 125 days of observation (data on day 125 not shown). HbA_{1C} is a marker for pathogenesis in type 2 diabetes[32], and this is increased to more than threefold compared to control. All these indicate that nutritionally induced insulin-resistant perch simulate the condition found in type 2 diabetes. Hence this could be used as a non mammalian model for type 2 diabetes. Another important point is the reversibility of nutritionally induced insulin resistance in perch. Withdrawal of palmitate feeding for 25 days showed a clear indication of reversal.

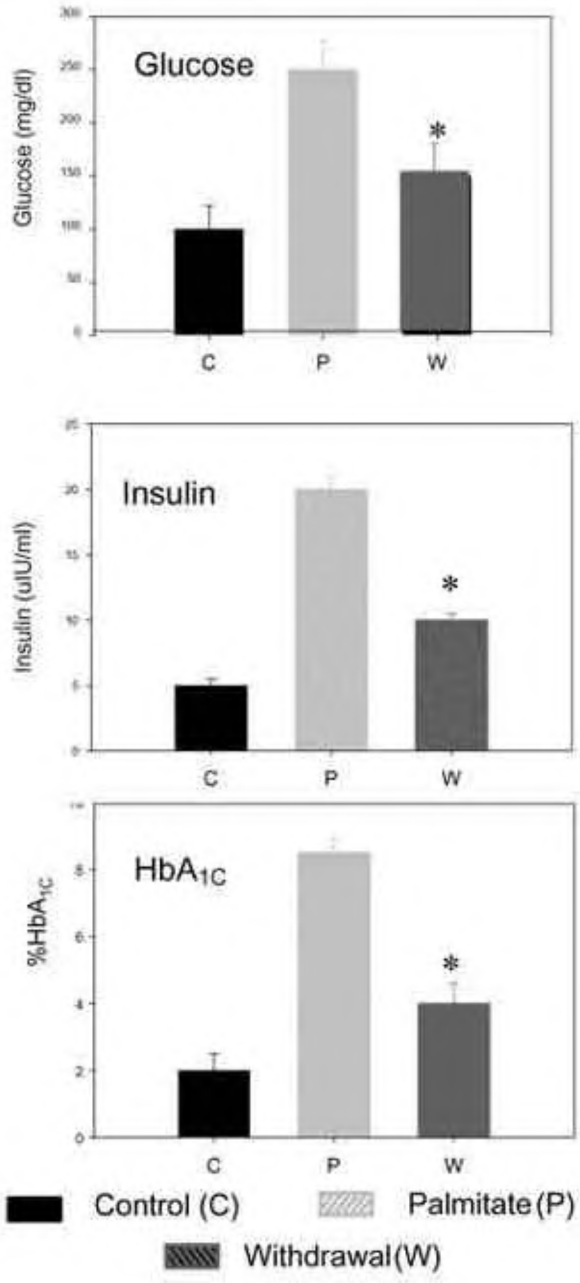

Figure 3. Withdrawal effect of palmitate on plasma glucose, insulin and HbA_{1C} levels. Blood samples were collected from perch fed with normal diet (C) or with usual diet plus palmitate (P) for 100 days or palmitate feeding was withdrawn for 25 days after 100 days of treatment (W). Plasma glucose, serum insulin and HbA_{1C} were determined from each group of experiments. All values expressed are ± SEm of three independent observations taking ten fishes for each observation. *Significance at $P < 0.01$ compared to palmitate-treated group.

We have selected palmitate to induce insulin resistance depending on several reports which demonstrate palmitate as the most potent FFA in inhibiting insulin activity[14,18–20]. Earlier studies also demonstrated that incubation of isolated muscle strips or cultured muscle cells with FFA strongly reduces insulin-stimulated glucose uptake, and this has been used regularly as a dependable means to examine the loss of insulin activity[14–17,33]. When we examined isolated skeletal muscle cells from control and palmitate treated perch for insulin-stimulated [^{3}H] 2-DOG uptake, a strong inhibition of [^{3}H] 2-DOG uptake occurred due to palmitate treatment. Magnitude of insulin-stimulated glucose uptake by fish skeletal muscle may not be the same as in mammals and human beings. Being a poikilothermic animal, requirement of energy by fish skeletal muscle should be comparatively lower than homeothermic animals. Fish belongs to the lowest group in the evolution of vertebrates, and insulin regulation of carbohydrate metabolism here may be expected to be less evolved than in mammals. However, this may not be a realistic interpretation since muscular activity in teleostean fish is appreciably high and some of the fish genomes (zebra fish and fugo fish) showed significant homology with the human genome. On this perspective, information obtained with nutritionally induced insulin-resistant perch may be important for future studies. Since lowering of glucose transport by FFA has been shown to be linked with insulin signalling defects[31,34], we investigated insulin-mediated signals starting from phosphorylation of insulin receptor tyrosine kinase. Phosphorylation of insulin receptor tyrosine kinase was dramatically reduced due to palmitate intake, suggesting that palmitate inhibition of [^{3}H] 2-DOG uptake is linked to insulin signalling cascade. We also examined other insulin augmented downstream signals which get consequently phosphorylated following receptor tyrosine kinase phosphorylation. Using phospho-specific antibodies we found that in the skeletal muscle cells of palmitate-fed perch, insulin stimulation of IRS1, PI-3K, PDK1 and Akt phosphorylation was significantly inhibited. Densitometric analysis of Western blots indicates two–three-fold decrease of insulin-stimulated phosphorylation in all these molecules. The closeness in the range of inhibition in palmitate-induced disruption of insulin signalling molecules from upstream to downstream, supports the physiological relevance of our study. We have observed a decrease in glucose uptake, PDK1 and Akt phosphorylation in FFA-fed perch skeletal muscle compared to skeletal muscle from control perch. Although there is a concern in extrapolating our observations directly to type 2 diabetes in humans, the insulin-resistant perch, within its limitations, could serve as a convenient model to evaluate the efficacy of various therapeutic agents targeting type 2 diabetes.

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