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Mechanistic population modeling of diabetes disease progression in Goto-Kakizaki rat muscle

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Abstract

Pyruvate dehydrogenase kinase 4 (PDK4) is a lipid status responsive gene involved in muscle fuel selection. Evidence is mounting in support of the therapeutic potential of PDK4 inhibitors to treat diabetes. Factors that regulate PDK4 mRNA expression include plasma corticosterone, insulin and free fatty acids. Our objective was to determine the impact of those plasma factors on PDK4 mRNA and to develop and validate a population mathematical model to differentiate aging, diet and disease effects on muscle PDK4 expression. The Goto-Kakizaki (GK) rat, a polygenic non-obese model of type 2 diabetes, was used as the diabetic animal model. We examined muscle PDK4 mRNA expression by real-time QRT-PCR. Groups of GK rats along with controls fed with either a normal or high fat diet were sacrificed at 4, 8, 12, 16, and 20 weeks of age. Plasma corticosterone, insulin and free fatty acid were measured. The proposed mechanism-based model successfully described the age, disease and diet effects and the relative contribution of these plasma regulators on PDK4 mRNA expression. Muscle growth reduced the PDK4 mRNA production rate by 14% per gram increase. High fat diet increased the initial production rate constant in GK rats by 2.19-fold. The model indicated that corticosterone had a moderate effect and PDK4 was more sensitive to free fatty acid than insulin fluxes, which was in good agreement with the literature data.

Keywords

population model; type 2 diabetes; disease progression; PDK4; Goto-Kakizaki rats

Introduction

Pyruvate Dehydrogenase Complex (PDC) catalyses the irreversible oxidation of pyruvate to yield acetyl-CoA and CO₂, and therefore is a key enzyme controlling the rate of oxidative glycolysis and regulating the balance between oxidation of carbohydrate and lipid fuels. The activity of PDC is controlled through its inactivating phosphorylation by pyruvate dehydrogenase kinases (PDKs) and activating dephosphorylation by pyruvate dehydrogenase phosphatases. PDK4, one of four identified isoforms, is regulated by a

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number of hormonal and nutritional factors, and it has been suggested that this enzyme, through its inactivation of PDC, is a key molecular switch governing fuel utilization [1].

Type 2 diabetes is associated with alterations in the balance between glucose and lipid pathways. Peripheral insulin resistance is a common feature of many type 2 diabetics. In addition, excessive hepatic glucose production, largely from gluconeogenesis, accounts for a significant proportion of hyperglycemia in some type 2 diabetics. Activation of PDC should act to mitigate diabetes by inhibiting gluconeogenesis and promoting glucose disposal in peripheral tissues [2]. There is some evidence supporting this since dichloroacetate, an inhibitor of PDK, has been previously used for treatment of several metabolic and cardiovascular disorders. However, chronic use of dichloroacetate is restrained due to its toxicity on liver and kidney, therefore it is not suitable for diabetes treatment [3–4].

Four distinct PDK isoenzymes have been identified [5–6]. PDK2 is ubiquitously expressed, PDK1 is restricted to the heart and PDK3 is largely restricted to the testis with low abundance. PDK4 is of particular interest for diabetes because it is expressed at high levels in heart and skeletal muscle which are major sites of glucose disposal, and to a lesser extent in liver. PDK4 is rapidly up-regulated in response to starvation [7] and is markedly up-regulated in skeletal muscle in animal models of type 1 diabetes [8] and steroid-induced diabetic rats [9–10].

Glucocorticoids are one hormonal factor which regulates PDK4 expression. PDK4 mRNA and protein are augmented in rats following dexamethasone treatment and are associated with decreased glucose oxidation rates [11]. Glucocorticoids activate PDK4 expression through glucocorticoid receptor (GR) mediated glucocorticoid receptor element (GRE) binding pathway. The induction was suppressed after a GR antagonist RU486 treatment [9]. Mutation of the glucocorticoid receptor element in the PDK4 promoter region also prevents PDK4 induction [12].

An additional hormone controlling PDK4 expression is insulin. Evidence suggests that insulin suppresses PDK4 mRNA expression in skeletal muscle and its effect is no dependent on the insulin effect on plasma free fatty acid [13]. The forkhead transcription factor forkhead box class O (FOXO) 1 has been implicated in the regulation of PDK4 expression by insulin [12]. Insulin signaling via protein kinase B (PKB) phosphorylates and enhances the translocation of FOXO factors from the nucleus to the cytoplasm, resulting in inhibition of the expression of genes that are up-regulated by FOXO factors. The FOXO factors bind to insulin response elements (IREs) [14] and their insulin directed removal from the nuclear compartment mediates the negative effects of insulin on gene expression [15]. An IRE has been identified within the rat PDK4 promoter region [16]. Insulin is no longer effective on human PDK4 after mutation of IREs [12]. Therefore, one possible mechanism of insulin suppressing PDK4 expression in skeletal muscle is to promote translocation of FOXO1 from nucleus to cytoplasm therefore decreasing FOXO1 activity in the nucleus.

High fat diet induced diabetes is known to lead to an increase in the flux of free fatty acids (FFA) into a variety of tissues. Fatty acids are known to regulate expression of a number of important metabolic enzymes [17]. One mechanism for this effect of fatty acids involves activation of peroxisome proliferator-activated receptors (PPARs) that control gene transcription in response to small lipophilic compounds [18]. Activation of PPAR α can promote PDK4 expression. WY-14,643, a synthetic PPAR α ligand, induced PDK4 expression in heart muscle of wild-type mice but not in PPAR α -null mice [19]. These results suggest that increases in free fatty acids can directly affect the level of PDK4 via PPAR α .

The development of type 2 diabetes involves many genetic and environmental factors, such as polygenic gene alterations and postnatal environmental factors (sedentary lifestyle and

unhealthy diet). The Goto-Kakizaki (GK) rat represents an animal model of non-insulin dependent diabetes with glucose intolerance due to potential impaired insulin secretion, insulin resistance and abnormal glucose metabolism [20–21]. In contrast to many other animal models of type 2 diabetes, GK rats don't become obese [20]. In the GK rat, moderate diabetes usually occurs between 3 and 4 weeks of age. Late complications include thickening of the glomerular and tubular basement membranes as well as glomerular hypertrophy. Therefore, this model provides a valuable tool for dissecting the pathogenesis of insulin resistance.

Modeling involves developing mathematical equations to describe quantitative relationships for individuals or populations. These equations can be used to predict the time course of disease and drug effects. The field of disease progression modeling has evolved greatly in the past few decades [22]. However, disease progression models tend to have high unexplained between subject variability (IIV), which is consistent with the observation that IIV is greater for pharmacodynamic than pharmacokinetic evaluations [23]. Nonlinear mixed effects models, also known as the hierarchical nonlinear models provide a tool for recognizing and estimating two distinct types of variability in the data: between-individual variability and within-individual variability. The relationship between the explanatory variable and the response variable is modeled as a single function but also allows the parameters to differ between individuals. NONMEM[®] is a program that allows model building to be performed using a population approach. One important feature of population analysis is the ability to describe between-subject variability, and to account quantitatively for covariate influences, such as weight, which can explain some of this variability [24]. Therefore the nonlinear mixed effects model is well suited for the analysis of biological data when observations are of the same biological category but the individual observations are different. The objective of this study was to investigate the dynamics of muscle type 2 diabetes (T2D) disease progression in normal-fed and high fat-fed Goto-Kakizaki (GK) rats and to develop a semi-mechanistic based population model for muscle PDK4 mRNA changes over time. The proposed model integrates information on the time course of the disease with effects of corticosterone, insulin, and free fatty acid on PDK4 mRNA expression into a single comprehensive, physiologically meaningful model structure.

Materials and Methods

Animals and Experimental design

This study involved 60 GK diabetic male rats and 59 WKY (Wistar-Kyoto) control male rats obtained from Taconic Farms (Germantown, NY, USA). The research protocol adheres to the 'Principles of Laboratory Animal Care' (NIH publication 85-23, revised in 1985) and was approved by the University at Buffalo Institutional Animal Care and Use Committee. Animals arrived at 21 ± 3 days of age. For experimental purposes, all animals were considered 22 days old at the time of arrival. At the time of arrival, each strain was randomly divided into 2 subgroups. Thirty GK and thirty WKY rats received a normal rat diet (ND) containing 10% energy from fat (Harlan TekLad 2016), while the remaining 30 GK and 29 WKY rats of each strain received a high fat diet (HF) containing 45% energy from fat (Harlan TekLad TD.06415). Animals were maintained in a separate room under stringent environmental conditions that included adherence to uninterrupted 12 h light: 12 h dark cycles. All animal care and manipulations were carried out between 1.5 and 3.5 h after lights on. Food intake and body weights of each animal were measured twice weekly. Six animals from each group were sacrificed at five different ages: 4, 8, 12, 16, and 20 weeks. However, due to receipt of 1 female animal, the WKY-HF-12 week group consisted of only 5 animals. Animals were anesthetized with ketamine (80 mg/kg)/valium (5 mg/kg) i.p. and killed by aortic exsanguination using EDTA (4 mM final concentration) as anticoagulant. Blood glucose was measured from whole blood at the time of killing. Plasma was prepared

from blood by centrifugation (2000 g, 4 °C, 15 min), divided into aliquotes, and stored at –80 °C. Gastrocnemius muscles were harvested from both legs, weighed, rapidly frozen in liquid nitrogen, and stored at –80 °C until analysis.

Glucose

Blood glucose was measured using a BD Logic blood glucose meter (BD Medical, Franklin Lakes, NJ, USA) from whole blood at sacrifice. Because the upper limit of detection of the blood glucose meter is 600 mg/dl, and some animals at later ages exceeded this limit, glucose was also measured in plasma following sacrifice. Plasma glucose was measured by the glucose oxidase method (Sigma GAGO-20). The manufacturer's instructions were modified such that the assay was carried out in a 1 ml assay volume, and a standard curve consisting of seven concentrations over a 16-fold range was prepared from the glucose standard and run with each experimental set. Experimental samples were run in triplicate.

Blood HbA1c

Blood HbA1c was measured by A1cNOW InView HbA1C test meters (Metrika, Sunnyvale, CA) from whole blood at sacrifice.

Plasma hormones

Insulin and corticosterone were measured in plasma samples using commercial RIAs (RI-13K Rat Insulin RIA Kit, Millipore Corporation, St Charles, MO; Corticosterone RIA Kit (rat and mouse), MP Biomedicals, Solon, OH). All assays were carried out according to the manufacturer's directions with standards run in duplicate and experimental samples run in triplicate.

Plasma Free Fatty Acid

The Roche Half-micro Test (Roche Applied Sciences, Indianapolis, IN) was modified to a microtitre plate format as follows: 200 ul Reagent A, 10 ul samples, 10 ul Reagent 3, 10 ul Reagent B, R2 incubation time of 60 minutes. All experimental samples were measured in triplicate. A standard curve consisting of 7 concentrations of FFA ranging from 0.05 – 1 uM was constructed from a commercial standard solution (WAKO NEFA, WAKO Chemicals, Richmond, VA). Linear regression analysis of all standard curves yielded r^2 values of greater than 0.99, and intra- and inter-assay variations were less than 10%.

RNA extraction

Frozen gastrocnemius muscles were ground into powder using a mortar and pestle chilled with liquid nitrogen. Total RNA extractions were carried out by a tri-reagent based extraction method. About 100 mg muscle powder from each animal was added to prechilled Trizol Reagent (Invitrogen, Carlsbad, CA) at a ratio of tissue: Trizol of 1:10. Extractions were performed according to manufacturer protocols. Before extraction, an external standard, glucose repressible gene (GRG) cRNA was added to each muscle sample to correct for variable extraction yields. Extracted RNA was further purified by passage through RNeasy mini-columns (Qiagen, Valencia, CA). Final extracted RNA samples were resuspended in nuclease-free water (Ambion, Austin, TX). Quantity of total RNA was determined by spectrophotometry and purity was assessed by agarose gel electrophoresis. Extracted total RNA preparations were diluted to desired concentration and stored at –80 °C until use.

Quantitative Real-Time Reverse Transcription Polymerase Chain Reaction

The yield of RNA extracted was determined for each muscle sample by comparing the quantity of exogenous GRG cRNA added into muscle tissue with that recovered after

extraction as previously described [25]. Target mRNA expression per gram tissue was calculated as the measured mRNA expression after extraction corrected to extraction yield. This procedure corrects for experimental variations in RNA yields between multiple tissue samples and allows conversion of mRNA expression to moles of message per gram wet weight of tissue. The quantity of GRG cRNA following extraction and muscle PDK4 mRNA were determined by quantitative real-time reverse transcription polymerase chain reaction (RT-PCR) using TAQMAN probes and gene specific in vitro transcribed cRNA standards for quantification. Primer and probe sequences were designed using PrimerExpress software (Applied Biosystems, Foster City, CA). Sequences sharing homology with other genes were excluded. The probe was labeled with reporter dye at the 5' end and a BHQ quencher at the 3' end and custom synthesized by Biosearch Technologies, Inc. (Novato, CA). The RT-PCR was performed using Brilliant® QRT-PCR Core Reagent Kit, 1-Step (Stratagene, La Jolla, CA) according to instructions of the manufacturer. A standard curve for each individual real-time RT-PCR assay was generated using in vitro transcribed sense cRNAs. The cRNA was constructed using conventional cloning methods and in vitro transcribed using T7 MEGAscript™ In Vitro Transcription Kits (Ambion, Austin, TX). For quantitation of PDK4 mRNA by real-time QRT-PCR the following were used: forward primer: 5'-AAGCCACATTGGGAGTATC-3', reverse primer: 5'-CAAAGGCATCTTCGACTA-3', and a FAM labeled probe (5'-CCCAACTGCGATGTGGTAGCA-3'). For quantitation of GRG cRNA, the following primers and probe were used: forward primer: 5'-CGGTTCTGGTGTAATGCTAAAGCT-3', reverse primer: 5'-AGTTCGCCAAGGGCTTCTC-3', and a HEX labeled probe: 5'-CCCTTCGAAATTCCAAGCCAAGTATGTCAT-3'). Average CVs were 5.4% for PDK4 and 6.4% for GRG.

Statistical analysis

Because of the large number of experimental samples, two samples were selected as “QC standards” for all assays to exclude possible experimental variations between different tests. Inter-assay variations of QCs were less than 10% for all assays. For statistical comparisons, two-way ANOVAs were carried out using SigmaStat 3.5 software (Systat Software, Point Richmond, CA) with Tukey post-hoc tests on rank-transformed data.

Data Analysis

Analysis of the data was carried out using NONMEM VI level 1.2 software for nonlinear mixed effects modeling [26]. Mixed effects modeling allows for the simultaneous analysis of data from multiple individuals while maintaining the correlation of observations within a given individual. The first order conditional estimation (FOCE) method was used throughout the model building using the subroutine ADVAN6 for the data analysis. Three significant digits were requested for the final parameter estimates. The tolerance of the differential equation solver used in the data analysis was set to 6.

Residual variability

To obtain homogeneity of the intra-individual (residual) error variance for the data analysis, an additive residual error model on the untransformed scale was used.

Interindividual variability

The inter-individual variability (IIV) model was assumed to follow a log-normal distribution using an exponential model.

Model discrimination

Model selection was based on physiological understanding of the PDK4 regulation, the objective function value (OFV) produced by NONMEM and graphical analysis using basic goodness-of-fit (GOF) plots of, for example, population predictions vs. observed concentrations, weighted residuals vs. observed concentrations and time.

Model description—The mechanism-based model includes corticosterone, insulin, and free fatty acid concentrations in addition to PDK4 mRNA levels (Fig. 1). PDK4 mRNA homeostasis was described by a complex indirect response model and is stimulated/inhibited by plasma factors. The production rate of PDK4 mRNA was closely related to the plasma concentrations of corticosterone, insulin and free fatty acid. During exploration of the plasma factors and PDK4 mRNA relationships, the combinations of signals were explored and the base model includes all three signals as time dependent covariates. Equation 1 describes the time profile of muscle PDK4 mRNA,

$$\frac{dPDK_{mRNA}}{dt} = k_{in} \bullet FDIS \bullet (1 + COAEF - COBEF + COCEF) - k_{out} \bullet PDK_{mRNA} \quad (1)$$

where PDK_{mRNA} is the PDK4 mRNA level, k_{in} is the PDK4 mRNA production rate constant and k_{out} is the PDK4 mRNA degradation rate constant. Corticosterone, insulin and free fatty acid effects on PDK4 mRNA production are described by COAEF, COBEF and COCEF respectively. A disease factor, FDIS, is also incorporated to describe the age effect. High fat fed WKY and GK rats have quite different initial PDK mRNA levels compared to normal fed controls and, for modeling purpose, the mRNA degradation rate constant was fixed. Therefore we include one disease factor, DIS3, to describe the difference between GK and WKY rat PDK production rate constants during the high fat feeding. k_{in} is modeled as:

$$\text{WKY RATS: } k_{in} = k_{in} \quad (2)$$

$$\text{GK RATS: } k_{in} = k_{in} \bullet (1 + DIS3) \quad (3)$$

Although our approach incorporated the hormonal regulation of PDK4 production to capture the disease effect on the PDK4 mRNA dynamics, the decrease in PDK4 mRNA expression with age was not fully described. Therefore, the age effect is described by a disease factor, FDIS. Individual disease factors for each strain and each diet were tested and the final model was constructed having only two different disease parameters to account for the difference with diet.

$$\text{NORMAL DIET RATS: } FDIS = \exp(-DIS1 \bullet t) \quad (4)$$

$$\text{HF DIET RATS: } FDIS = \exp(-DIS2 \bullet t) \quad (5)$$

Corticosterone effects on PDK4 production were modeled as a nonlinear function [9] on k_{in} as:

$$COAEF = \frac{S_{\max} \bullet COA}{SC_{50} + COA} \quad (6)$$

Insulin's effect on PDK mRNA was described in equation (7). Insulin is assumed to control PDK4 mRNA by inhibiting its production. Change in insulin from its initial value (COBASE) can inhibit PDK4 production with a linear efficiency constant COBG.

$$COBEF = COBG \bullet (COB - COBASE) \quad (7)$$

Free fatty acids are also a major regulator of PDK4 mRNA production. Changes in FFA from its initial value (COCBAS) were assumed to stimulate PDK4 mRNA production with a linear efficiency constant COCG.

$$COCEF = COCG \bullet (COC - COCBAS) \quad (8)$$

Covariate Analysis

The covariate analysis was performed using an automated method [27]. The effects of plasma glucose and muscle weight on PDK4 mRNA disease progression were analyzed. After fitting the base model, empirical Bayesian estimates (EBEs) of the inter-individual random effects were computed and plotted against all available covariates to detect relationships between parameters and covariates. The effect of each covariate on each parameter was statistically tested through inclusion in the population model. The most significant covariate effect was incorporated into the population model to form the new base model. The new base model was used to compute EBEs and relationships between parameters and covariates were examined again. This process was repeated until there were no significant covariates remaining in the population model (full multivariable model). Subsequently, the most non-significant covariate was excluded from the full multivariable model and this process was then repeated until all remaining covariates in the model were significant. The likelihood ratio test (LRT) was used to compare the fit of covariate models. The difference in the minimum value of the objective function ($\Delta MVOF$) has a χ^2 distribution with the degrees of freedom equal to the change in parameter numbers. A change in MVOF of ≥ 7.88 and 10.83 was required to reach statistical significance at $p \leq 0.005$ and $p \leq 0.001$ for the inclusion or elimination of one fixed effect. In addition, the improvement of the fit in the covariate model was also evaluated from the change in the inter-individual variability and residual variability. Other graphic evaluations included scatter plots of weighted residuals against predicted concentrations as well as weighted residuals against time.

Model evaluation

A non-parametric bootstrap analysis was performed as an internal model evaluation technique, using the software package Wings for NONMEM (N. Holford, Version 404, June 2003, Auckland, New Zealand). A new replication of the original dataset (a bootstrap sample) was obtained by N random draws of individual animal data (with replacement) from the original dataset, and the full model was re-fitted to each new dataset. This process was repeated 1000 times with different random draws and the stability of the full model was evaluated by visual inspection of the distribution of the model parameter estimates from the new datasets and compared with that obtained from the fit of the combined dataset [28].

Bootstrap runs where the minimization was not successful were excluded from further analysis. The final model parameter estimates were compared to the mean and 95% confidence intervals (CI) of the non-parametric bootstrap replicates of the final model.

Results

Subjects and data

In total, 119 rats were included in the disease progression study. The demographic characteristics of the study population are summarized in Table 1. Initially, 6 WKY rats were included in the 12 week control rats high fat diet fed group. However, 1 rat was identified as female and therefore data from that rat was excluded from analysis. Fig 2 shows the expression profiles of plasma corticosterone, insulin and free fatty acid concentrations in our study. When fed a normal diet, muscles from diabetic GK rats had significantly higher PDK4 mRNA expression compared with control rats from 4 weeks old to 12 weeks old. High fat feeding elevated PDK4 mRNA in both strains at all age groups except for 20 week in WKY rats. Plasma corticosterone concentrations were significantly higher at all ages in GK compared with WKY under normal diet. However, the high fat feeding only elevated corticosterone at 4 week old in GK and 16 week in WKY compared with the normal fed strains. Under normal diet feeding, plasma insulin concentrations in the control rats increased modestly between 4 and 8 weeks old and remained at a relatively constant level throughout 20 week experiment period. However, insulin increased dramatically in the GK population between 4 and 8 weeks, remained at this elevated level through 12 weeks then began to decline such that by the end of the experimental period plasma insulin was marginally higher in control than in GK rats. High fat feeding had little effect on the insulin concentrations in either strain. There was no significant difference in plasma free fatty acid concentration between GK and control rats under normal diet. High fat diets had a large impact in both strains. The free fatty acid concentration was higher from 4 week to 12 week in high fat fed GK. The high fat diet elevated the free fatty acid concentration in control rats from 8 week to the end of the experiment period.

Disease Progression Model Diagnostics

Results from our study are reported and interpreted in the form of nonlinear mixed-effects or “population” analysis. Plots of observed muscle PDK4 mRNA versus population predictions (PRED) are shown in Fig 3 [29]. They clearly demonstrate the adequacy of the final model to describe the observed dynamics data of PDK4 mRNA in our GK study. The performance of the final model is also diagnosed by typical goodness-of-fit plots (Fig 4a–c). The goodness-of-fit plots of observations versus population predictions give a useful impression of the extent of variability in the data that is explained by structure components of the model. Evidence of a good fit is the even spread of the data around the line of identity. The plot of weighted residuals vs. PRED and the plot of weighted residuals vs. time provides diagnostics for the residual error model. In both figures, the weighted residuals show a horizontal scatter indicating adequacy of handling the residual error distribution.

Model evaluation

Internal validation of the model was conducted by means of a non-parametric bootstrap technique. The distribution of the THETA, OMEGA, and SIGMA parameter estimates obtained from fitting many bootstrap replications are used to generate confidence intervals for parameters and to explore model robustness. From the 1000 bootstrap replicates 133 failed to minimize successfully. When the mean values of all parameters resulting from the successful and combined (successful and unsuccessful) bootstrap replicates were compared, the difference was less than 1%. Also, when the 95% confidence limits for all parameters resulting from the successful and combined bootstrap estimates were compared, the

difference was lower than 5%, except for the upper limit of insulin sensitivity on k_{in} (13%). This suggested that a number of unsuccessful minimizations in the bootstrap analysis may be due to an inaccurate estimation of the insulin sensitivity (COBG). This could possibly be explained by the fact that the insulin profile was very complex and the individual variability of insulin concentration was large. For these reasons, the unsuccessful bootstrap replicates were considered unreliable and hence excluded from further analysis. The results of the 867 successful bootstrap replicates are given in Table 4.

The population estimates obtained by fitting the final model to the data were very similar to the mean of the 867 bootstrap replicates and were contained within the respective 95% confidence intervals, suggesting a high accuracy of the NONMEM parameter estimates. The precision of the NONMEM parameter estimates was good; since the standard error of estimate (RSE) was lower than 60% for the fixed and random effects, with the notable exception of the insulin sensitivity (COBG) and residual variability. The lack of precision in the estimation of residual variability is possibly related to the fact that there are limited subjects and sampling points and the overall PDK4 mRNA data variability is large. The inclusion of the covariate effects in the final model based on the LRT was confirmed by the 95% confidence intervals for the covariate effect parameters obtained from the bootstrap replicates.

Model Analysis

The base model was fitted successfully to the dynamic data of PDK4 except for the high fat diet diabetic GK rats group. The population parameter estimates for the base model obtained are presented in Table 2. The delta plots identified muscle weight and glucose concentration as potential covariates on k_{in} and Dis1. The covariate analysis was performed using an automated method. The final model retained only muscle weight on k_{in} (Table 3). The final model improved the fit relative to the base model ($\Delta MVOF = 15.488$, d.f. = 1, $p < 0.001$). The interindividual variability for k_{in} and Dis1 were reduced 5.5 and 9.3, respectively. The resulting parameter estimates are presented in Table 4. The corticosterone maximal stimulation effect of PDK4 mRNA was characterized as 0.686, indicating corticosterone has a moderate effect on PDK4 mRNA production. The PDK4 mRNA production was substantially more sensitive to free fatty acid (5.18 mM^{-1}) than insulin (0.0158 ml/ng). The dynamic change of PDK4 mRNA production under normal diet was estimated at $e^{0.00841 \cdot t}$, indicating the production rate constant decreased from 0.96-fold at 4 weeks to 0.84-fold at 20 weeks. The dynamics of PDK4 mRNA production with high fat diet was estimated at $e^{-0.01 \cdot t}$, indicating the production rate constant increased from 1.04-fold at 4 weeks to 1.22-fold at 20 weeks. The Dis3 was characterized as 1.19; therefore the high fat diet also increased the initial PDK4 mRNA production in GK rats by 2.19-fold. There was a negative muscle weight effect on the k_{in} . In fact, a 1 gram increase in the muscle weight corresponded to a 14% decrease in k_{in} .

Discussion

The development of type 2 diabetes involves many genetic and environmental factors, such as polygenic gene alterations, prenatal environmental alterations (maternal malnutrition [30] or exposure to corticosteroids in the third trimester [31]) and postnatal environmental factors (sedentary lifestyle and unhealthy diet) [32–33]. The multi-factorial interactions make the progression of type 2 diabetes complex. Dissecting the multiple interacting factors should provide insight into the effects of individual factors involved in type 2 diabetes progression.

PDK4 is the major enzyme responsible for the inhibition of PDC activity in muscle. PDC links glycolysis with several biochemical pathways. PDC catalyzes oxidative decarboxylation of pyruvate to form acetyl-CoA and provides the carbon units for the

complete oxidation in the citric acid cycle. Enhanced PDK4 activity reduces glucose uptake into the muscle in insulin resistance states [34]. PDK4 inhibitors are a promising therapeutic approach for type 2 diabetes, evidenced by the use of dichloroacetate in several metabolic and cardiovascular disorders. PDK4 mRNA was elevated in the muscle in Goto-Kakizaki rats, a spontaneous diabetic animal model. The expression of PDK4 mRNA was also increased in both GK and control rats after high fat feeding. Relatively large variability has been observed in PDK4 mRNA expression level. Therefore, population disease progression modeling was applied to dissect the variability and to quantify the disease progression and diet effects on PDK4 mRNA dynamics by evaluating the impact of plasma corticosterone, insulin and free fatty acid on PDK4 mRNA.

The population disease progression model includes a linear function of muscle weight on the production rate of PDK4 mRNA. In the current analysis, the PDK4 mRNA production rate was reduced 14% per gram increase in muscle weight. The age effect on PDK4 mRNA was characterized by Dis1 where the coefficient of k_{in} dropped from 0.96 to 0.84 during the 20 week experimental period. In the current analysis, the diet effect on the dynamics of PDK4 mRNA was characterized by Dis2 where the coefficient of k_{in} increased from 1.04 to 1.22 during the 20 week experimental period. High fat feeding had more pronounced effects on diabetic GK rats where the high fat diet increased the initial production rate constant by 2.19-fold as indicating by the parameter estimation of Dis3. These findings were expected based on the known effect of high fat diet on promoting systemic metabolic disturbances [35]. It is further supported by the fact that elevated PDK4 mRNA and activity were shown in high fat fed Wistar rat muscle [36]. Three plasma regulators were investigated and incorporated as the time dependant covariates to account for the disease progression effect on PDK4 mRNA expression. Corticosterone, a major regulator of gluconeogenesis, has been shown to enhance PDK4 gene expression through glucocorticoid receptor mediated processes. Plasma free fatty acids mediate PDK4 expression through PPAR α , while insulin is able to inhibit PDK4 expression, possibly due to the promotion of FOXO translocation from nucleus to cytoplasm. Based on model estimates and simulation results, PDK4 mRNA was more sensitive to free fatty acid and insulin, and less sensitive to corticosterone. This is in good agreement with in vitro studies as well [9].

The performance of the final model in describing data is diagnosed by typical goodness-of-fit plots (Fig 4a–c). They demonstrate the adequacy of the final model to describe the observed dynamics of PDK4 mRNA expression in this study. However, due to limited subject numbers and sampling times, the model exhibits the perfect fit phenomenon where the variance of individual parameter estimates (EBE) distribution is shrinking towards zero and the individual weighted residuals (IWRES) distribution shrinks towards zero. The calculated epsilon shrinkage is 52% while the η -shrinkage for k_{in} and Dis1/Dis2 are 24% and 64%, respectively. Therefore EBE-based diagnostics lack informativeness and may be misleading, and are excluded from the goodness-of-fit plots.

The bootstrap analysis clearly demonstrated the accuracy and precision of the parameter estimates except for insulin sensitivity and residual error. The poor estimate of the insulin sensitivity is possibly due to the variability of the insulin data as well the complex pattern of the insulin concentration time profiles. In WKY animals, insulin increased modestly between 4 and 8 weeks of age and remained relatively constant throughout 20 weeks. In contrast, insulin increased dramatically (about 6-fold) in the GK population between 4 and 8 weeks, remained at this elevated level through 12 weeks, then began to decline such that by 20 weeks plasma insulin was marginally higher in WKY than in GK, possibly due to pancreatic failure during the later ages [37]. In addition, a large residual error is expected since the inter-subject variability of PDK4 mRNA is generally high especially with high fat

feeding (CV% 's of 30%–50%). This is likely due to the inherent variability of the animal model.

Conclusion

A population approach was employed to integrate the time course of disease utilizing plasma corticosterone, insulin and free fatty acid concentrations to describe the dynamic changes of muscle PDK4 mRNA. The disease progression resulted in an increase of PDK4 mRNA expression in diabetic GK rats. High fat feeding resulted in an increase of PDK4 mRNA and had a more profound effect on the initial production of PDK4 mRNA in diabetic GK rats. This study also revealed a negative age effect on PDK4 mRNA levels, partially due to muscle growth. The developed model of muscle PDK4 mRNA dynamics was able to characterize the PDK4 mRNA dynamics in diabetic GK and control rats under both normal and high fat feeding and provides a scientific support for PDK4 as a biomarker for type 2 diabetes disease progression. Our model also extends our understanding of diabetes disease progression and high fat effects on muscle into a quantitative relationship to possibly predict the time course of disease. In addition, our model could be further used for testing anti-diabetic drug effects which target muscle glucose utilization.

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Figure 1.
Model diagram for PDK mRNA. Symbols are defined in text and Table 2.

Figure 2.
Observed plasma corticosterone, insulin and free fatty acid concentrations.

Figure 3. Population predictions (lines) and observed muscle PDK4 mRNA vs. age for GK and WKY rats.

Figure 4. Scatter plots of the observed muscle PDK4 mRNA vs. the population model predictions (A), the population weighted residuals vs. the population model predictions (B) and the population weighted residuals vs. age (C)

Table 1

Characteristics of GK and WKY rats.

Characteristic	mean	median	range	N
Muscle weight (g)	3.16	3.29	0.52–6.38	120
Plasma corticosterone (ng/ml)	242	215	24.3–683	120
Plasma insulin (ng/ml)	0.1	0.09	0.03–0.24	120
Plasma free fatty acid (mM)	6.79	6.08	2.1–24.65	120

N: sample size.

Table 2

Population disease progression parameter estimates for PDK4 dynamics (base model).

Parameter Estimate			Description
Parameter (units)	Population Mean	%SEM	
k_{in} (fmol/gram/week)	122	17.5	WKY rat PDK production rate constant
k_{out} (1/week)	77	NE	PDK degradation rate constant
S_{max}	1.38	47	corticosterone maximal stimulation of PDK4 production
SC_{50} (ng/ml)	346	NE ⁹	corticosterone concentration causing half-maximal stimulation of PDK4
COBG(ml/ng)	0.022	53.9	insulin sensitivity of PDK production
COBASE	1.19/2.63	NE	initial insulin concentration of WKY/GK rats
COCG(1/mM)	4.89	31.3	FFA sensitivity of PDK production
COCBAS	0.06/0.1	NE	initial FFA concentration of WKY/GK rats
DIS1	0.007	32.2	disease progression effect on PDK production rate under Normal diet feeding
DIS2	-0.01	NE ^a	disease progression effect on PDK production under high fat diet feeding
DIS3	1.1	19.5	High fat diet effect on initial PDK production rate constant in GK rats
IIV k_{in} (%CV)	33.6	34.3	inter-subject variability of k_{in}
IIV DIS1/DIS2(%CV)	273	52.5	inter-subject variability of dis1/dis2
RV	0.273	94.6	residual variability

NE: not estimated;

⁹ Refer to reference 9;^a based on fitting results from model development process.

Table 3

Summary of the covariate analysis for the disease progression analysis of muscle PDK4.

parameter	covariate	functional form	change in OFV	degree of freedom	p-value
k_{in}	MW	linear	-18.5	1	0.0000171
DIS1	MW	linear	-16.3	1	0.0000548
DIS1	MW	exponential	-14.7	1	0.000124
DIS1	GLU	linear	-14.5	1	0.000137
k_{in}	GLU	exponential	-0.638	1	0.424
k_{in}	GLU	linear	-0.608	1	0.436

Table 4

Disease progression parameter estimates for PDK4 dynamics (final model).

Structure Parameter (units)	Original dataset		867 Bootstrap replicates		
	Estimate ^a	%SEM	Mean ^b	95%CI Lower limit	95%CI Upper limit
k_{in} (fmol/gram/week)	128	14.6	129	125	131
k_{out} (1/week)	77	NE	NE	NE	NE
S_{max}	0.686	63.3	0.742	0.612	0.87
SC_{50} (ng/ml)	346	NE	NE	NE	NE
COBG (ml/ng)	0.0158	848.1	0.00635	0.00329	0.00941
COBASE	1.2/2.6	NE	NE	NE	NE
COCG (1/mM)	5.18	26.6	5.23	5.11	5.4
COCBAS	0.06/0.1	NE	NE	NE	NE
DIS1	0.00841	31.5	0.00882	0.00825	0.00941
DIS2	-0.01	NE	NE	NE	NE
DIS3	1.19	16.7	1.13	1.07	1.22
MW	-17.4	27.8	-16.9	-17.9	-15.9
Interindividual variability (%)					
IIV k_{in} (%CV)	28.1	40.1	27.2	26.5	28.2
IIV DIS1/DIS2 (%CV)	264	57.5	243.6	237.1	250.2
Residual variability					
RV	0.292	94.3	32.5	31.7	33.7

^aTypical population parameter estimate of final model for the original dataset.

^bMean value of the population parameters of 867 bootstrap replicates (minimization not successful for 133 replicates)