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Insulin Downregulates Pyruvate Dehydrogenase Kinase (PDK) mRNA: Potential Mechanism Contributing to Increased Lipid Oxidation in Insulin-Resistant Subjects[1]

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Oxidative metabolism of glucose is regulated by pyruvate dehydrogenase (PDH) that can be inhibited by isoforms of PDH kinase (PDK). Recently, increased PDK activity has been implicated in the pathogenesis of insulin resistance and non-insulin-dependent diabetes mellitus (NIDDM) in obese subjects. Using quantitative RT-PCR, we measured mRNA of PDK2 and PDK4 isoforms in skeletal muscle biopsies from nondiabetic Pima Indians, a population with a high prevalence of NIDDM associated with obesity. PDK2 and PDK4 mRNAs were positively correlated with fasting plasma insulin concentration, 2-h plasma insulin concentration in response to oral glucose, and percentage body fat, whereas both isoforms were negatively correlated with insulin-mediated glucose uptake rates. Measurements of PDK2 and PDK4 mRNA during the hyperinsulinemic-euglycemic clamp and of PDK2 in cell culture indicated that both transcripts decrease in response to insulin. Increased fatty acid (FA) oxidation has been traditionally viewed as the cause for increased PDK activity contributing to insulin resistance in obese subjects. In contrast, our data indicate that insufficient downregulation of PDK mRNA in insulin-resistant individuals could be a cause of increased PDK expression leading to impaired glucose oxidation followed by increased FA oxidation.

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Pima Indians of Arizona have the highest known prevalence of non-insulin-dependent diabetes mellitus (NIDDM) in the world (1,2), and insulin resistance and obesity are strong risk factors for the development of the disease (3,4). In 1963, Randle proposed a competition between glucose and fatty acid oxidation (the glucose/fatty acid cycle) as the biochemical basis for insulin resistance and potentially for NIDDM (5). Subsequently, it was demonstrated that the oxidation of lipids and glucose are inversely related (6–11), and that obese subjects with or without NIDDM oxidize more fat than lean subjects (12,13). Furthermore, increased lipid oxidation reduces the ability of skeletal muscle to store glucose as glycogen (10,11,13–15), consistent with a decreased glycogen synthase activity in this tissue that is typically found in obese subjects and patients with NIDDM (16–18). Moreover, pharmacologic inhibition of lipid oxidation in obese individuals leads to an enhanced glucose disposal and storage (19), thus providing supportive evidence for a direct link between changes in lipid metabolism and alterations in glucose utilization.

Recent studies indicated that the inhibitory effect of fatty acids on glucose metabolism is mediated mainly through changes in the activity of pyruvate dehydrogenase kinase [PDK (7,8)]. This enzyme selectively inhibits pyruvate dehydrogenase (PDH), a mitochondrial enzymatic complex that catalyzes the

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first irreversible step in glucose oxidation (reviewed in 7,8). A rapid activation of PDK in response to fatty acids (FAs) is caused primarily by an increase in acetyl-CoA and NADH, whereas a longer term effect of FAs is associated with an increased specific activity of PDK (7,8). Based on these studies, it has been proposed that activation of PDK caused by increased lipid/FA oxidation may play a primary role in the development of insulin resistance and NIDDM in obese individuals (7,8).

Four distinct PDK isoenzymes encoded by separate genes are known in human (20,21), including PDK4 identified by positional cloning in the chromosomal region 7q21.3 that is possibly linked with insulin resistance and NIDDM in the Pima Indians (21,22). Although we did not detect any alterations in the coding sequences of PDK4 in a previous study (21), information on its genomic structure and tissue expression enabled us to quantify mRNA of this isoform in skeletal muscle biopsies collected from nondiabetic Pima Indians. For comparison, we also measured mRNA of PDK2, the most prevalent isoform in human tissues (20), and investigated the effect of insulin on PDK expression in skeletal muscle and in a human rhabdomyosarcoma cell line. We show that treatment with insulin *in vivo,* as well as in a cell line system under conditions that approximate physiological metabolic environment, correlates with a decrease in PDK mRNA, and we conclude that inadequate downregulation of PDK mRNA could be a cause for the elevated PDK activity in insulin-resistant subjects. We speculate that increased FA oxidation accompanied by inhibition of glucose oxidation may be a consequence, rather than the cause, of impaired insulin action.

SUBJECTS AND METHODS

Subjects

The subjects are members of the Gila River Indian Community who have been participating in studies of NIDDM since 1965 (2,4). Nondiabetic volunteers were admitted to the clinical research ward for 7–10 days and fed a weight-maintaining diet. Each participant underwent several tests on different days, including a 75-g oral glucose tolerance test (OGTT), underwater weighing to determine body composition including percentage body fat (PFAT), a two-step hyperinsulinemic-euglycemic clamp combined with indirect calorimetry to simultaneously determine insulin-mediated whole body glucose uptake ($IMGU_l$ and $IMGU_h$ in response to low-dose and high-dose of insulin, respectively), glucose oxidation and storage, and respiratory quotient (RQ) (4). Further clinical parameters utilized in this study include fasting plasma insulin level (FPI) and 2-h plasma insulin level measured during OGTT (2HPI). In addition, skeletal muscle biopsies were collected from several participants after an overnight fast (23), making it possible to assess differences in gene expression between insulin-resistant and insulin-sensitive subjects in the basal state, and without the potentially confounding effects of biochemical changes caused by overt diabetes. The *in vivo* effect of insulin on PDK mRNA concentration was measured in skeletal muscle biopsies collected from 13 subjects (8 males, 5 females) before and after a 100-min one-step hyperinsulinemic-euglycemic clamp (24) using 3600 $pmol/m^2/min$ of insulin. Human subject studies were approved by the Institutional Review Board of the National Institute for Diabetes and Digestive and Kidney Diseases, and the Tribal Council of the Gila River Community. All volunteers gave a written consent after being informed about the nature and possible consequences of the studies.

Skeletal Muscle Biopsies, RNA, and Protein Isolation

Collection of percutaneous skeletal muscle biopsies from m. vastus lateralis and extraction of total RNA and protein were performed as described previously (23).

Rhabdomyosarcoma Cell Culture

Cell line A204, derived from a human rhabdomyosarcoma, was obtained from the American Type Culture Collection (ATCC, Rockville, MD; Catalog No. HTB 82). Cells were cultured in Dulbecco's modified Eagle medium (D-MEM with high glucose, Cat. No. 11995-032; Gibco-BRL, Gaithersburg, MD) supplemented with 10% fetal calf serum (Gibco-BRL). For starvation/refeeding experiments, D-MEM was substituted by serum- and glucose-free basal incubation medium [IM medium, (25)] at approximately 80% cell confluence. After 20 h, the medium was replaced by fresh IM supplemented with 5.5 mmol/L glucose and 100 nmol/L porcine insulin (Sigma Chemical Co., St. Louis, MO), and replicate cultures were harvested after 60- and 120-min intervals. Control cells were treated identically, except that insulin was omitted. The effect of a 20-h starvation in IM was

TABLE 1

Clinical Profile of 36 Nondiabetic Pima Indians (29 males/7 females) Selected for PDK RNA Expression Study

	Mean ± SD
Age (years)	30 ± 1
BMI (kg/m^2)	33.6 ± 7.4
PFAT	32 ± 7
Fasting plasma glucose (mmol/L)	4.7 ± 0.6
$IMGU_L$ (mg/min · kg $EMBS^{-1}$)	2.9 ± 0.9
$IMGU_H$ (mg/min · kg $EMBS^{-1}$)	8.1 ± 2.3
Log_{10} FPI (pmol/L)	2.4 ± 1.0
Log_{10} 2HPI (pmol/L)	3.0 ± 1.2

Note. BMI, body mass index; PFAT, percentage body fat; $IMGU_L$ [$IMGU_H$], low-dose [high-dose] insulin-mediated whole body glucose uptake rate; EMBS, estimated metabolic body size; FPI, fasting plasma insulin; 2HPI, 2 h plasma insulin during oral glucose tolerance test (OGTT).

assessed by comparison with cells cultured in D-MEM.

Quantitative Reverse Transcription PCR (RT-PCR)

Preparation of cDNA from total RNA and quantification of the PDK4 transcript were described in (21,23), except that TaqStart antibody (Clontech Laboratories, Inc., Palo Alto, CA) was used for hot start PCR according to the manufacturer's instructions. RT-PCR of the PDK2 transcript was performed with the sense primer 5′-GCCATGAGTCCAGCCTCATTC and antisense primer 5′-GACAGGCAGGCGCTCCACC, duplexed with amplimers for either β-actin or G3PDH (Clontech Laboratories) used as internal standards for mRNA quantification. PCR-amplified products were resolved on ethidium bromide-stained agarose gels, quantified by scanning densitometry of the negative image of each gel (23), and all experiments were performed at least in triplicate.

Western Blotting

Muscle extracts (100 μg of protein per lane) were separated on 10% SDS-PAGE and transferred to a nitrocellulose membrane. Blots were probed with a rabbit polyclonal antibody (diluted 1:500) raised against the recombinant rat PDK2, followed by ^{125}I-labeled protein A (DuPont NEN, Boston, MA) (23). To facilitate comparison between blots, a replica of the same sample and 20 ng of purified recombinant PDK4 (21) were included on each gel. The Western blot analysis was performed with a subset of 26 subjects from which a sufficient amount of the biopsied material was available, and all protein samples were analyzed at least in triplicate.

Statistical Analyses

The statistical significance of correlations between quantitative clinical parameters, mRNA, and immunoreactive protein was assessed using the Statistical Analysis Systems program package (SAS, Cary, NC). Differences of PDK2 and PDK4 mRNA concentrations in skeletal muscle biopsies collected before and after the hyperinsulinemic-euglycemic clamp and data from experiments with the rhabdomyosarcoma cell line were analyzed by paired Student's t test.

RESULTS

Quantification of PDK2 and PDK4 mRNA in Basal State

Table 1 summarizes clinical characteristics of all 36 nondiabetic subjects included in this study. As shown in Table 2, we found a negative correlation of the mRNA level for both PDK isoforms with $IMGU_L$, and $IMGU_H$ (PDK2, $P < 0.03$ and $P = 0.01$, respectively; PDK4, $P = 0.03$ and $P < 0.04$, respectively), and a positive correlation with FPI and 2HPI (PDK2, $P = 0.0003$ and $P = 0.009$, respectively; PDK4, $P = 0.03$ and $P = 0.002$, respectively). We also found a negative correlation of PDK4 transcript with basal glucose oxidation ($P < 0.02$), and basal RQ ($P = 0.04$). In addition, both mRNAs were positively correlated with PFAT, although only the result with PDK2 was statistically significant ($P =$

TABLE 2

Correlation of Clinical Parameters with PDK2 and PDK4 mRNA Concentration in Skeletal Muscle of Nondiabetic Pima Indians ($N = 36$)

Clinical parameter	PDK2 mRNA		PDK4 mRNA	
	r	*P*	*r*	*P*
PFAT	0.56	(0.002)	0.52	(0.066)
$IMGU_L$	−0.37	(0.026)	0.36	(0.032)
$IMGU_H$	−0.42	(0.012)	0.35	(0.039)
Log_{10} FPI	0.58	(0.0003)	0.37	(0.033)
Log_{10} 2HPI	0.45	(0.009)	0.51	(0.002)

Note. Log_{10} FPI, $logarithm_{10}$ of fasting plasma insulin concentration; Log_{10} 2HPI, $logarithm_{10}$ of 2-h plasma insulin concentration during OGTT. See Table 1 for remaining abbreviations.

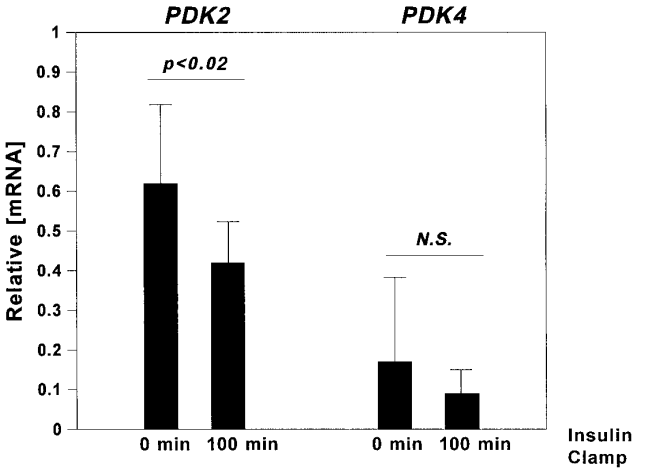

FIG. 1. Insulin response of PDK2 and PDK4 mRNA in skeletal muscle of nondiabetic Pima Indians. Skeletal muscle biopsies were collected before and after 100 min of one-step hyperinsulinemic-euglycemic clamp (insulin dose 3600 pmol/m^2/min) in 13 Pima Indians (8 males and 5 females); PDK2 and PDK4 mRNA was quantified by RT-PCR in relation to β-actin mRNA. Data are presented as means ± SD.

0.002). The negative correlations of PDK2 and PDK4 with FPI and of PDK4 with 2HI remained statistically significant after adjustment for PFAT (PDK2/FPI, $P < 0.03$; PDK4/FPI, $P = 0.03$, PDK4/2HPI, $P = 0.004$).

In Vivo Effect of Insulin on PDK2 and PDK4 mRNA

To assess whether insulin could have an *in vivo* effect on PDK expression, both mRNAs were quantified in skeletal muscle biopsies collected from 13 Pima Indians before and after a 100-min infusion of a high dose of insulin (3600 pmol/m^2/min). In response to this treatment, the relative level of PDK2 transcript decreased by 32% ($P < 0.02$; Fig. 1), and that of PDK4 by 47%, although the latter difference was not statistically significant presumably due to a larger interindividual variation in the measurements of this transcript. However, the insulin-mediated mRNA changes correlated significantly between the two isoforms ($r = 0.78$, $P = 0.002$).

In Vitro Effect of Insulin on PDK2 mRNA

The effect of insulin on PDK2 mRNA was also investigated in the human rhabdomyosarcoma cell line A204. This analysis was limited to PDK2, because we were unable to detect expression of PDK4 in these cells. As shown in Fig. 2, incubation of the cells for 20 h in a serum- and glucose-free medium ("starved" condition) resulted in approximately 3-fold increase of PDK2 mRNA when compared with cells maintained in complete medium ("fed" condition; $P < 0.01$). Treatment of starved cells with 100 nmol/L insulin plus 5.5 mmol/L glucose for 60 and 120 min (that should simulate the conditions achieved *in vivo* during the hyperinsulinemic-euglycemic clamp) led to a significant decrease of PDK2 mRNA ($P < 0.05$ and $P < 0.01$, respectively), which returned to the level observed in the fed control (Fig. 2).

Quantitative Analysis of PDK Protein in Skeletal Muscle

To examine the relationship between PDK2 mRNA and protein in the basal state, we measured immunoreactive PDK by Western blot analysis in skeletal muscle extracts from 26 of the 36 subjects included in this study. We used a polyclonal antibody against the recombinant rat PDK2 and detected in all individuals a ~46-kDa band consistent with the size expected for human PDK2 (20). Although the result was indicative of a weak positive correlation between the immunoreactive protein

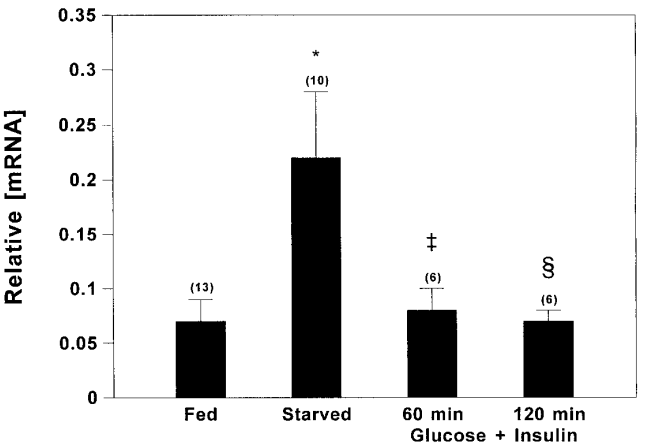

FIG. 2. Changes of PDK2 mRNA in A204 cells caused by starvation, and in response to treatment with insulin plus glucose. The effects of a 20-h incubation in serum- and glucose-free medium (Starved), followed by treatment with 100 nmol/L insulin plus 5.5 mmol/L glucose for 60 and 120 min (60 min and 120 min, respectively), were compared with control cells maintained in complete D-MEM medium (Fed). Data represent means ± SD calculated from at least two separate experiments, representing a total number of replications indicated in parentheses above each bar. *$P < 0.01$, when compared to Fed. ‡, §$P < 0.05$ and $P < 0.01$, respectively, when compared to Starved.

and PDK2 mRNA ($r = 0.36$), this relationship did not reach statistical significance ($P = 0.08$).

DISCUSSION

The present study was initiated to complement a detailed analysis of the PDK4 gene at 7q21.3, which we found to be possibly linked with insulin resistance and NIDDM in the Pima Indians (21). We have previously reported that we did not detect any alteration(s) in the coding sequences of PDK4 that would explain this linkage (21), and we have now compared mRNA concentration for the PDK4 and PDK2 isoforms in skeletal muscle from insulin-resistant and insulin-sensitive Pima Indians. We found correlations of both isoforms with obesity (PFAT), as well as with parameters of insulin action, including a positive correlation with FPI and 2HPI, and a negative correlation with $IMGU_L$, $IMGU_H$. Furthermore, PDK2 and PDK4 mRNA decreased in response to insulin during a hyperinsulinemic-euglycemic clamp, and a similar change of PDK2 mRNA was also observed in an insulin-treated human rhabdomyosarcoma cell line. These data indicate that the concentration of PDK2 and PDK4 transcripts can be modified by insulin.

Insulin can regulate the expression of some genes via promoter-specific sequences (e.g., 26,27), including recently described DNA elements involved in insulin-mediated repression of transcription of phosphoenolpyruvate carboxykinase (PEPCK) (26). We have previously determined about 4 kb of the 5′ flanking genomic sequence in PDK4 (21), but none of the reported, insulin-responsive promoter elements are present in this locus. We predict, however, that the expression of PDK2 and PDK4 mRNAs could be regulated by a common mechanism, because both transcripts show comparable correlations with parameters of insulin action and respond similarly to insulin. Further functional studies will be required to determine how insulin regulates PDK mRNA.

The lack of a statistically significant correlation between the PDK2 protein and mRNA could be explained by a lower specificity of the antiserum, developed originally against the rat PDK2 that is 90% identical with the human counterpart (20). The antiserum also cross-reacted with the purified human recombinant PDK4 control run on each blot (not shown). Because the molecular size of PDK4 [~46.5 kDa (21)] is similar to PDK2, we could not differentiate between the relative contributions of each isoform to the intensity of the immunoreactive band in skeletal muscle extracts, and it is possible that this antibody may recognize epitopes on all four human PDK isoforms which share 62–69% amino acid residues (20,21). The immunoreactive band measured in skeletal muscle may therefore represent total PDK protein, and this could obscure any potentially isoform-specific correlation when compared with the transcript of only one gene, such as PDK2. Development of specific antibodies will be necessary to explore a biologically relevant relationship between mRNA and protein for each isoform.

The role of Randle's glucose/FA cycle in the pathogenesis of insulin resistance and NIDDM is supported by human studies involving insulin/glucose clamps with indirect calorimetry (6,10,17), combined infusions of lipid/heparin plus insulin (14), and treatment with β-pyridylcarbinol that inhibits fatty acid oxidation (19). These studies demonstrated an inverse relationship between the metabolism of fatty acids and insulin-mediated regulation of glucose metabolism. Recent biochemical findings indicate an important pathogenic role of PDK in the glucose/FA cycle, and it has been postulated that increased PDK activity in obese individuals will contribute to insulin resistance and development of NIDDM (7,8).

So far, all predictions about the pathogenic role of PDK were based on measurements of enzymatic activity and protein level in cultured cells or in tissue samples. To our knowledge, this is the first study to analyze the regulation of two human PDK isoforms at the mRNA level, providing evidence that their expression is modified by insulin.

Although our data do not provide insights into the molecular mechanism(s) that mediate the effect of insulin on PDK mRNA, the finding that insulin-resistant Pima Indians do not downregulate the expression of both PDK isoforms despite hyperinsulinemia could indicate a novel mechanism by which PDK may contribute to altered glucose metabolism in obese subjects with impaired insulin action. The traditional concept of Randle's hypothesis assumes that increased lipid/FA oxidation is the primary biochemical abnormality leading to an activation of PDK, which contributes to impaired glucose metabolism through an inhibition of PDH. However, our findings offer an alternate hypothesis for viewing abnormal interactions between glucose and FA metabolism in insulin-resistant individuals. We speculate that insufficient insulin-mediated suppression of PDK gene expression in skeletal muscle could be a cause for increased PDK protein and enzymatic activity in insulin-resistant subjects. The ensuing

inhibition of glucose oxidation would be compensated for by an increased FA oxidation to maintain intracellular concentrations of acetyl-CoA and $NADH_2$.

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