Insulin suppresses PDK-4 expression in skeletal muscle independently of plasma FFA

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Submitted 13 October 2003; accepted in final form 9 March 2004

Lee, Felix N., Lihuah Zhang, Dan Zheng, Woo S. Choi, and Jang H. Youn. Insulin suppresses PDK-4 expression in skeletal muscle independently of plasma FFA. Am J Physiol Endocrinol Metab 287: E69–E74, 2004. First published March 16, 2004; 10.1152/ajpendo.00461.2003.—Starvation and experimental diabetes induce a stable increase in pyruvate dehydrogenase kinase (PDK) activity in skeletal muscle, which is largely due to a selective upregulation of PDK-4 expression. Increased free fatty acid (FFA) level has been suggested to be responsible for the upregulation. Because these metabolic states are also characterized by insulin deficiency, the present study was designed to examine whether insulin has a significant effect to regulate PDK mRNA expression in rat skeletal muscle. In study 1, overnight-fasted rats received an infusion of saline or insulin for 5 h (n = 6 each). During the insulin infusion, plasma glucose was clamped at basal levels (euglycemic hyperinsulinemic clamp). A third group (n = 6) received Intralipid infusion during the clamp to prevent a fall in plasma FFA. PDK-2 mRNA level in gastrocnemius muscle was not altered by insulin or FFA (i.e., Intralipid infusion). In contrast, PDK-4 mRNA level was decreased 72% by insulin (P < 0.05), and Intralipid infusion prevented only 20% of the decrease. PDK-4 protein level was decreased ~20% by insulin (P < 0.05), but this effect was not altered by Intralipid infusion. In study 2, overnight-fasted rats were refed or received an infusion of saline or nicotinic acid (NA, 30 μmol/h) for 5 h (n = 5 each). During the refeeding and NA infusion, plasma FFA levels were similarly (i.e., 60–70% vs. saline control) lowered. Muscle PDK-4 mRNA level decreased 77% after the refeeding (P < 0.05) but not after the NA infusion. In conclusion, the present data indicate that insulin had a profound effect to suppress PDK-4 expression in skeletal muscle and that, contrary to previous suggestions, circulating FFA had little impact on PDK-4 mRNA expression, at least within 5 h.

pyruvate dehydrogenase kinase; free fatty acid; insulin resistance; forkhead transcription factor

LIPID OXIDATION IS GENERALLY INCREASED in individuals with obesity or type 2 diabetes (3, 4, 17). Increased lipid oxidation suppresses glucose oxidation, and this substrate competition mechanism may play a major role in the development of insulin resistance (i.e., decreased insulin’s action on glucose uptake) in skeletal muscle (20, 21, 28). One major target for this regulation is the activity of pyruvate dehydrogenase (PDH) complex that catalyzes a key irreversible step of glucose oxidation, i.e., the conversion of pyruvate to acetyl-CoA. The activity of PDH complex is regulated acutely by the products of free fatty acid (FFA) oxidation, i.e., acetyl-CoA and NADH. In addition, PDH complex is inhibited by phosphorylation of its PDH component by pyruvate dehydrogenase kinase (PDK).

Increased PDK activity has been suggested to play an important role in suppressing glucose oxidation and inducing insulin resistance in individuals with obesity and type 2 diabetes (20, 21).

Previous studies have shown that starvation and experimental diabetes induce a stable increase in PDK activity in skeletal muscle (5, 7, 25), which explains the decreased activity of PDH and reduced glucose oxidation in these metabolic states. Skeletal muscle expresses two (i.e., PDK-2 and PDK-4) of the four PDK isoforms expressed in mammalian cells (PDK-1–4) (10, 24). The increase in PDK activity with starvation and diabetes was shown to be largely due to a selective upregulation of PDK-4 expression (29, 30). Because the plasma FFA level increases with starvation and diabetes, FFA has been suggested to stimulate PDK-4 expression in skeletal muscle (21, 28); FFA is an endogenous ligand for the peroxisome proliferator-activated receptor-α (PPARα) (6, 9) and may increase PDK-4 expression by stimulating PPARα (11, 30). However, there are also large changes in other substrates and hormones in the blood during starvation or with diabetes. For example, the plasma insulin level decreases dramatically with starvation or diabetes. Insulin is known to have a profound effect to increase glucose oxidation, and it is therefore possible that the increase in PDK-4 expression (and decreased glucose oxidation) with starvation and diabetes is, at least in part, explained by insulin deficiency (5, 15, 30). However, few studies have examined directly whether insulin has a regulatory effect on PDK-4 expression in skeletal muscle. Major et al. (15) showed that a hyperinsulinemic euglycemic clamp resulted in decreases in PDK mRNA levels in human skeletal muscle, suggesting a direct effect of insulin on PDK expression. However, this study, which employed a short exposure (i.e., 100 min) to insulin failed to demonstrate statistical significance for a small decrease in PDK-4 mRNA level while demonstrating a significant decrease in PDK-2 mRNA. Also, because plasma FFA level falls during hyperinsulinemic glucose clamps, the possibility cannot be excluded that insulin decreased PDK expression indirectly by decreasing plasma FFA. One goal of the present study was to examine whether insulin has a significant effect to regulate PDK-4 mRNA expression in rat skeletal muscle independently of plasma FFA. Another goal was to test whether changes in plasma FFA (in the absence of changes in plasma insulin) induced by an infusion of nicotinic acid (NA) (26) can alter PDK-4 mRNA expression in skeletal muscle.
METHODS

Animals and catheterization. Male Wistar rats weighing 275–300 g were obtained from Simonsen (Gilroy, CA) and studied ≥5 days after arrival. Animals were housed under controlled temperature (22 ± 2°C) and lighting (12-h light, 0600–1800; 12-h dark, 1800–0600) with free access to water and standard rat chow. At least 4 days before the experiment, animals were placed in individual cages with tail restraints as previously described (1, 2, 13), which was required to protect tail blood vessel catheters during experiments. Animals were free to move about and were allowed unrestricted access to food and water. Two tail vein infusion catheters were placed the day before the experiment, and one tail artery blood sampling catheter was placed ≥4 h before the experiment (i.e., ~0700). All procedures were approved by the Institutional Animal Care and Use Committee at the University of Southern California.

Experimental protocols. Experiments were conducted after an overnight fast; food was removed at 1700 on the day before the experiment. Two studies were carried out. In study 1, we determined the effects of plasma insulin, with and without Intralipid infusion to prevent a fall in plasma FFA, on skeletal muscle PDK-2 and PDK-4 mRNA expression. Animals received an infusion of porcine insulin (Eli Lilly, Indianapolis, IN) at a constant rate of 30 pmol·kg⁻¹·min⁻¹ for 5 h. During the insulin infusion, plasma glucose was clamped at basal levels by exogenous glucose infusion (hyperinsulinemic euglycemic clamp). The hyperinsulinemic euglycemic clamp was performed with (n = 6) or without (n = 6) a simultaneous infusion of Intralipid [Liposyn II (Abbott, North Chicago, IL); triglyceride emulsion, 20% wt/vol; 0.9 ml/h] and heparin (40 U/h, with 10 U as a priming bolus) (2). A third group of rats received only saline infusion (i.e., no insulin infusion; n = 6) for 5 h. At the end of the saline infusion or clamps, animals were anesthetized, and gastrocnemius muscles were rapidly dissected out, frozen immediately using liquid N₂-cooled aluminum blocks, and stored at −80°C for later analysis for PDK expression. In study 2, we tested in overnight-fasted rats the effects of refeeding and NA-induced decreases in plasma FFA on skeletal muscle PDK-2 and PDK-4 mRNA expression. One group of animals (n = 5) was refed 5 h, and another group (n = 5) received a constant infusion of NA [30 µmol/h (26)] for 5 h to decrease plasma FFA similarly to the refed animals. During the NA infusion, small amounts of glucose were infused to prevent a fall in plasma glucose (26). A third group received only saline infusion (n = 5) for 5 h to serve as controls. At the end of the 5-h saline or NA infusion or refeeding, animals were anesthetized, and gastrocnemius muscles were taken as in study 1. In all of these experiments, blood samples were taken at various time points to measure plasma glucose, insulin, and FFA.

Northern blot analysis. Total RNA was extracted from frozen muscles using Tri Reagent from Molecular Research Center (Cincinnati, OH) according to the manufacturer’s instructions. The total RNA preparations (25 µg each) were then loaded onto a 1% denaturing agarose gel, and electrophoresis was performed with 1× MOPS running buffer (Ambion, Austin, TX) at 50 V for 3.5 h. RNA was then capillary transferred overnight onto a positively charged nylon membrane (BrightStar-Plus, Ambion). cDNA probes for rat PDK-2 and PDK-4 were obtained by RT-PCR using an Advantage One Step RT-PCR kit from Clontech (Palo Alto, CA) and the following primers: PDK-4, 5’-CGTGGCCAAGATTAAGGCTC-3’; PDK-2, 5’-GTCAGTGGGGGCCTTCCTTTC-3’; and 5’-CAGGACTGAGGCAGGATGA. The cDNA probes were labeled with [³²P]dCTP (Perkin Elmer) using a DECAprime DNA labeling kit (Ambion). Hybridization was carried out using Ultraserve solution (Ambion). After overnight hybridization at 42°C with the cDNA probes, the blots were then washed twice in 2× SSC (sodium chloride and sodium citrate buffer) for 15 min and twice in 0.1× SSC for 15 min. The autoradiograph was developed on Kodak BioMax MS film. Relative densities were quantified using the Bio-Rad Molecular Analyst. To control RNA loading, all blots were quantified for glyceraldehyde-3-phosphate dehydrogenase (G3PDH) by use of a probe included in the DECAprime DNA labeling kit (Ambion).

Western blot analysis. Frozen muscles (50 mg) were homogenized using a polytron at half-maximum speed (1 min, on ice) in 500 µl of buffer (20 mM Tris, pH 7.5, 5 mM EDTA, 10 mM NaH₂PO₄, 100 mM NaF, 2 mM Na₂VO₃, 1% NP-40, 1 mM PMSF, 10 µg/ml aprotinin, and 10 µg/ml leupeptin). Muscle lysates were further solubilized by continuous stirring (4°C, 1 h) and centrifuged (14,000 g, 20 min). The supernatants (50 µg) were resolved by SDS-PAGE followed by electrophoretic transfer of proteins onto Hybond-P membranes (Amersham, Piscataway, NJ). The membranes were then probed with a rabbit antiserum (1:1,000) against PDK-4 (generous gift from Dr. R. A. Harris, Indiana University School of Medicine, Indianapolis, IN) and a secondary antibody (horseradish peroxidase-conjugated anti-rabbit IgG, Amersham). Signals were detected by an enhanced chemiluminescence method and quantified using the Bio-Rad Molecular Analyst.

Other assays. Plasma glucose was analyzed by a glucose oxidase method on a Beckman Glucose Analyzer II (Beckman, Fullerton, CA). Plasma insulin was measured by radioimmunoassay using a kit from Linco Research (St. Charles, MO). Plasma FFA was measured using an acyl-CoA oxidase-based colorimetric kit (Wako Pure Chemical Industries, Osaka, Japan).

Statistical analysis. Data are expressed as means ± SE. The significance of the differences in mean values among different treatment groups was evaluated using one-way ANOVA followed by ad hoc analysis using the Tukey test. P < 0.05 was considered statistically significant.

RESULTS

Plasma insulin, glucose, and FFA levels during hyperinsulinemic euglycemic clamp with or without simultaneous infusion of Intralipid (study 1). Insulin infusion raised plasma insulin to ~600 pM, and this level was slightly increased by Intralipid infusion (P < 0.05; Fig. 1). During the insulin infusion, plasma glucose was clamped at basal levels (~6.0 mM). Plasma FFA levels decreased ~65% during insulin infusion (P < 0.05), and this was prevented by Intralipid infusion. In fact, plasma FFA levels increased above basal during the combined infusion of insulin and Intralipid (P < 0.05). The glucose infusion rate (GINF) required to clamp plasma glucose, reflecting insulin’s action to suppress hepatic glucose production and increase peripheral glucose uptake, increased rapidly during the 1st h and reached steady state within 2 h of initiation of the clamps. Elevation of plasma FFA via Intralipid infusion decreased GINF >50% at the end of the 5-h clamp (P < 0.05).

Effects of insulin and FFA on PDK-4 mRNA and protein levels in skeletal muscle. The PDK-4 mRNA level in gastrocnemius muscle was decreased dramatically by insulin (Fig. 2). Elevation of plasma FFA during insulin infusion had a small effect to increase PDK-4 mRNA expression (P < 0.05) but did not prevent the suppression of PDK-4 mRNA expression by insulin. Thus insulin had a profound effect to suppress PDK-4 mRNA expression independently of its effect to decrease plasma FFA levels. In contrast to the changes in PDK-4 mRNA, the PDK-2 mRNA level was not altered by insulin or FFA. The effect of insulin to decrease PDK-4 mRNA was also observed in soleus muscle (Fig. 3). Thus the insulin regulation of PDK-4 mRNA expression took place in both oxidative (i.e., soleus) and glycolytic (i.e., gastrocnemius) muscles. Insulin
also decreased PDK-4 protein content in gastrocnemius muscle (P < 0.05; Fig. 4). However, these changes in PDK-4 protein expression were relatively smaller than those in mRNA expression, probably because the 5-h insulin infusion period was not a sufficient time for decreased PDK-4 transcription to be fully reflected in protein level.

Plasma insulin, glucose, and FFA levels during refeeding or NA infusion (study 2). To test the role of circulating FFA in the regulation of PDK-4 mRNA expression in skeletal muscle during fasting and refeeding, overnight-fasted rats were refed or received a constant NA or saline infusion for 5 h. Refeeding raised plasma glucose and insulin throughout the 5-h refeeding period (P < 0.05 for both; Fig. 5). Plasma FFA decreased 60%, from 0.56 to 0.22 mM, within 1 h of the refeeding and remained suppressed during the rest of the refeeding period (P < 0.05). During the NA infusion, small amounts of glucose were infused to prevent a fall in plasma glucose, and plasma glucose was not different between the saline controls and the NA-infused rats. NA infusion did not alter plasma insulin. As expected, the NA infusion lowered plasma FFA levels (P < 0.05), and these changes in plasma FFA during the NA infusion were similar to (or larger than) those during the refeeding.

Fig. 1. Plasma insulin (A), glucose (B), and free fatty acid (FFA; C) concentrations and glucose infusion rate (GINF; D) during the 300-min saline infusion (●) or hyperinsulinemic euglycemic clamp with (○) or without (□) Intralipid infusion. Values are means ± SE for 6 experiments.

Fig. 2. Effects of insulin and Intralipid (FFA) infusions on pyruvate dehydrogenase kinase (PDK) mRNA expression in gastrocnemius muscle. A: representative Northern blots for PDK-2, PDK-4, and glyceraldehyde 3-phosphate dehydrogenase (G3PDH) mRNA expression in rats infused with saline (Basal), insulin (Insulin), or insulin and Intralipid (Insulin + FFA). B: Summary of PDK-2 and PDK-4 mRNA expression, normalized to G3PDH, for the 3 experimental groups. Values are means ± SE for 6 experiments. *P < 0.05 vs. basal; #P < 0.05 vs. insulin.

Fig. 3. Effects of insulin on PDK-4 and PDK-2 mRNA expression in soleus and gastrocnemius muscles. Northern blot analysis was performed on muscles of rats infused with saline or insulin for 5 h (n = 3 each).
Effects of refeeding and NA infusion on PDK-4 mRNA and protein levels in skeletal muscle. As reported by previous studies (30), refeeding of fasted rats markedly decreased PDK-4 mRNA level in gastrocnemius muscle (\( P < 0.05 \); Fig. 6). However, despite similar decreases in plasma FFA level, NA infusion did not alter muscle PDK-4 mRNA expression, indicating that changes in circulating FFA during refeeding cannot account for the rapid and profound decreases in muscle PDK-4 mRNA expression. In contrast, a 5-h insulin infusion caused a 72% decrease in muscle PDK-4 mRNA level, suggesting that insulin might be responsible for the changes in PDK-4 expression observed during refeeding. However, insulin concentrations during the insulin infusion (Fig. 1) were higher than those observed during the refeeding (Fig. 5); it is unclear whether insulin alone can fully account for the changes in PDK-4 expression during fasting and refeeding. Nonetheless, the present data clearly indicate that insulin has greater impact on skeletal muscle PDK-4 mRNA expression than FFA at least within the time frame (5 h) used in the present study.

**DISCUSSION**

The present study demonstrates a strong effect of insulin to suppress PDK-4 mRNA expression in skeletal muscle independently of its effect to decrease plasma FFA levels. The present data also indicate that plasma FFA had little impact on muscle PDK-4 mRNA expression, contrary to previous suggestions (21, 28). Thus muscle PDK-4 mRNA expression appears to be regulated by insulin rather than FFA. These findings suggest that the dramatic increase in PDK-4 expression observed with starvation or with diabetic animals may be largely due to insulin deficiency rather than to increased circulating FFA.

Previous studies have shown that fasting induced a two- to fivefold increase in PDK-4 mRNA and protein expression in skeletal muscle (11, 18, 29, 30) and that these fasting effects were rapidly reversed during refeeding (29). Changes in FFA concentration during fasting and refeeding have been suggested to be responsible for this regulation (21, 28). In the present study a 5-h refeeding of overnight-fasted rats decreased muscle PDK-4 mRNA level by 77% (completely reversing the fasting-induced increases; Fig. 7), which was associated with a profound suppression of plasma FFA. However, a similar suppression of plasma FFA induced by NA infusion failed to alter muscle PDK-4 mRNA level. These data indicate that the changes in circulating FFA during refeeding cannot account for the rapid and profound decreases in muscle PDK-4 mRNA expression. In contrast, a 5-h insulin infusion caused a 72% decrease in muscle PDK-4 mRNA level, suggesting that insulin might be responsible for the changes in PDK-4 expression observed during refeeding. However, insulin concentrations during the insulin infusion (Fig. 1) were higher than those observed during the refeeding (Fig. 5); it is unclear whether insulin alone can fully account for the changes in PDK-4 expression during fasting and refeeding. Nonetheless, the present data clearly indicate that insulin has greater impact on skeletal muscle PDK-4 mRNA expression than FFA at least within the time frame (5 h) used in the present study.

**Fig. 4.** Effects of insulin on PDK-4 protein expression in gastrocnemius muscles. A: representative Western blots for PDK-4 protein expression in rats infused with saline (Basal), insulin (Insulin), or insulin and Intralipid (Insulin + FFA). B: summary of PDK-4 protein expression for the 3 experimental groups. Values are means ± SE for 5 experiments. *\( P < 0.05 \) vs. basal.

**Fig. 5.** Plasma glucose (A), insulin (B), and FFA (C) concentrations during the refeeding (●) or saline (○) or NA (●) infusion for 5 h. Values are means ± SE for 5 experiments.

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*AJP-Endocrinol Metab • VOL 287 • JULY 2004 • www.ajpendo.org*
PPARα has been suggested to play a major role in the regulation of PDK-4 expression with starvation or diabetes. First, WY-14643, a PPARα agonist, was shown to increase PDK-4 expression in rat gastrocnemius (fast glycolytic) muscle (30). Because FFA is an endogenous ligand for PPARα (6, 9) and FFA levels increase with starvation or diabetes, these data suggest that PFK-4 expression may be increased in these metabolic states as a result of increased PPARα stimulation by FFA. To support this concept, the upregulation of PDK-4 expression in hepatic, renal, and cardiac tissues associated with starvation was abolished (31) or attenuated (27) in PPARα-null mice. However, a more recent study (11) showed that the upregulation of PDK-4 expression with starvation was retained in oxidative muscles (soleus and tibialis anterior) of PPARα-null mice (unlike those observed in the fast glycolytic gastrocnemius muscles in earlier studies), indicating the presence of PPARα-independent mechanisms for regulation of PDK expression in these muscles. Also, it should be pointed out that PPARα-null mice, compared with control mice, showed substantially altered response to starvation (in terms of circulating substrate and hormone levels) (11). For example, we noted that the decrease in insulin during starvation was significantly less in PPARα-null mice compared with control mice, providing a possible explanation for attenuated PDK-4 response to starvation in some of previous studies with PPARα-null mice (27, 31).

Recent evidence indicates that PDK-4 is a target gene of the forkhead transcription factor Foxo1 (8, 14). Also, Akt/PKB, which is stimulated by insulin, has been shown to phosphorylate Foxo1 and cause its translocation from the nucleus into the cytosol, resulting in reduced transcriptional activity in transfected 293 cells (22, 23). Taken together, these data support the possibility that insulin suppresses PDK-4 expression in skeletal muscle by decreasing Foxo1 activity in the nucleus. In addition, Foxo1 gene expression was suggested to play a role in the regulation of PDK-4 gene expression (8). A more recent study (16) showed that insulin-induced phosphorylation and nuclear export of Foxo1 increased ubiquitination-mediated degradation of Foxo1 in Hep G2 cells. Our preliminary data show that Foxo1 content in gastrocnemius muscles significantly increased with overnight fasting and decreased after the 5-h insulin stimulation (data not shown). Whether insulin indeed regulates Foxo1 phosphorylation, translocation, and/or expression in skeletal muscle and, if so, whether it is causally related to the regulation of PDK-4 expression remain to be tested.

If insulin plays a major role in regulating muscle PDK-4 expression, it is tempting to speculate on the possibility that insulin’s action to suppress PDK-4 expression is decreased in insulin-resistant states, which would result in increased PDK expression, leading to impaired glucose oxidation (15).
ous studies (12, 19) have shown that PDK-4 mRNA and protein expression increased in skeletal muscle with high-fat feeding, but it could not be inferred whether the increase was due to increased availability of fat or to a possible decrease in insulin’s effect on PDK-4 expression in the insulin-resistant state. This important issue regarding the role of insulin resistance on skeletal muscle PDK-4 expression warrants further investigation.

In conclusion, our results indicate that insulin had a dramatic effect to suppress PDK-4 (but not PDK-2) mRNA expression in skeletal muscle independently of insulin’s effect to decrease plasma FFA levels. In addition, and contrary to general belief, circulating FFA had little impact on PDK-4 mRNA expression in skeletal muscle. Our data suggest that the rapid and profound changes in PDK-4 mRNA expression during fasting or refeding (or experimental diabetes) might be largely due to changes in circulating insulin rather than FFA.

ACKNOWLEDGMENTS

We thank Drs. Joyce Richey and Chin K. Sung for insightful comments on the manuscript.

GRANTS

This study was supported by a research grant from the American Diabetes Association.

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