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Transcriptional regulation of pyruvate dehydrogenase kinase 4 in skeletal muscle during and after exercise

Henriette Pilegaard[1]* and P. Darrell Neufer[2]
[1]*Copenhagen Muscle Research Centre, August Krogh Institute, University of Copenhagen, Universitetsparken 13, 2100 Copenhagen Ø, Denmark*
[2]*John B. Pierce Laboratory and Department of Cellular and Molecular Physiology, Yale University, School of Medicine, New Haven, CT 06519, USA*

The pyruvate dehydrogenase complex (PDC) has a key position in skeletal muscle metabolism as it represents the entry of carbohydrate-derived fuel into the mitochondria for oxidation. PDC is regulated by a phosphorylation–dephosphorylation cycle, in which the pyruvate dehydrogenase kinase (PDK) phosphorylates and inactivates the complex. PDK exists in four isoforms, of which the PDK4 isoform is predominantly expressed in skeletal and heart muscle. PDK4 transcription and PDK4 mRNA are markedly increased in human skeletal muscle during prolonged exercise and after both short-term high-intensity and prolonged low-intensity exercise. The exercise-induced transcriptional response of PDK4 is enhanced when muscle glycogen is lowered before the exercise, and intake of a low-carbohydrate high-fat diet during recovery from exercise results in increased transcription and mRNA content of PDK4 when compared with intake of a high-carbohydrate diet. The activity of pyruvate dehydrogenase (PDH) is increased during the first 2 h of low-intensity exercise, followed by a decrease towards resting levels, which is in line with the possibility that the increased PDK4 expressed influences the PDH activity already during prolonged exercise. PDK4 expression is also increased in response to fasting and a high-fat diet. Thus, increased PDK4 expression when carbohydrate availability is low seems to contribute to the sparing of carbohydrates by preventing carbohydrate oxidation. The impact of substrate availability on PDK4 expression during recovery from exercise also underlines the high metabolic priority given to replenishing muscle glycogen stores and re-establishing intracellular homeostasis after exercise.

Pyruvate dehydrogenase kinase 4: Transcriptional regulation: Skeletal muscle: Exercise

Oxidative phosphorylation is crucial to skeletal muscle metabolism, particularly during prolonged endurance exercise. For oxidative use, carbohydrates enter the mitochondria via the decarboxylation of pyruvate to acetyl-CoA in a reaction catalysed by the pyruvate dehydrogenase complex (PDC):

$$\text{pyruvate} + \text{CoA} + \text{NAD}^+ \xrightarrow{\text{PDC}} \text{acetyl-CoA} + \text{CO}_2 + \text{NADH}.$$

This irreversible enzymic reaction plays a key role in muscle metabolism, as it represents the only entry point for carbohydrates (derived either from circulating glucose or intramuscular glycogen) into the mitochondria for complete oxidation. Thus, the PDC is positioned such that it is likely to play a key role in regulating the metabolic fate of glucose as well as overall substrate selection in skeletal muscle.

Structure and regulation of the pyruvate dehydrogenase complex

The mammalian PDC is a multienzyme assembly composed of multiple copies of three catalytic enzymes (pyruvate dehydrogenase (E1); dihydrolipoamide acetyltransferase (E2); dihydrolipoamide dehydrogenase (E3)), one structural protein (E3-binding protein) and two regulatory enzymes (pyruvate dehydrogenase kinase (PDK);

Abbreviations: E1, E2, E3, catalytic subunits of PDC; FKHR, forkhead homologue in rhabdomyosarcoma; PDC, pyruvate dehydrogenase complex; PDH, pyruvate dehydrogenase; PDK, pyruvate dehydrogenase kinase.

***Corresponding author:** Dr Henriette Pilegaard, fax +45 35 32 1567, email hpilegaard@aki.ku.dk

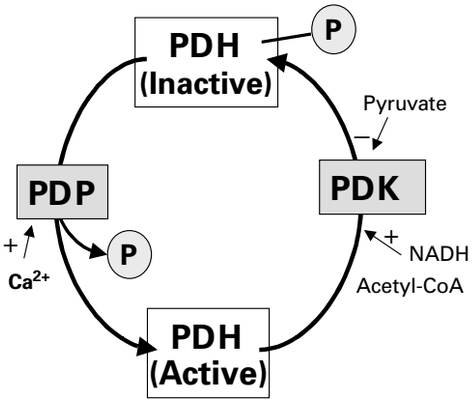

Fig. 1. Regulation of the pyruvate dehydrogenase complex (PDC) by phosphorylation–dephosphorylation. Phosphorylation by pyruvate dehydrogenase kinase (PDK) renders the PDC inactive, whereas dephosphorylation and reactivation of PDC is catalysed by pyruvate dehydrogenase phosphatase (PDP). PDH, pyruvate dehydrogenase.

pyruvate dehydrogenase phosphatase). Covalent modification via phosphorylation–dephosphorylation of the E1 component is thought to be the major mechanism regulating the enzyme. Phosphorylation of specific serine residues within the α subunit of E1 is catalysed by PDK rendering the enzyme inactive, whereas dephosphorylation and activation of PDC is catalysed by pyruvate dehydrogenase phosphatase (Fig. 1; for review, see Harris *et al*. 2001; Patel & Korotchkina, 2001; Reed, 2001; Roche *et al*. 2001; Sugden & Holness, 2003).

Early work revealed that PDK is inhibited allosterically by high concentrations of pyruvate (generated by glycolysis), whereas the kinase is activated by high mitochondrial acetyl-CoA:CoA and $NADH:NAD^+$ (typically arising with increased rates of fatty acid β-oxidation) through changes in the acetylation and reduction state of the E2 component of the PDC. Two pyruvate dehydrogenase phosphatase isozymes have been identified, both of which require Mg^{2+} for activity. Pyruvate dehydrogenase phosphatase 1 is predominantly expressed in skeletal muscle and is stimulated by physiological Ca^{2+} concentrations (for review, see Harris *et al*. 2001; Patel & Korotchkina, 2001; Reed, 2001; Roche *et al*. 2001; Sugden & Holness, 2003).

Evidence that PDC is also subject to long-term regulation has come from studies in which it was found that starvation and diabetes in rats induce a delayed but persistent increase in PDK activity without any corresponding increase in acetyl-CoA:CoA or $NADH:NAD^+$ (for review, see Harris *et al*. 2001; Patel & Korotchkina, 2001; Reed, 2001; Roche *et al*. 2001; Sugden & Holness, 2003). Deciphering the mechanisms responsible for this long-term regulation proved difficult until recent cloning studies revealed the existence of four distinct isoforms of PDK (PDK1–4) in mammalian tissues. PDK1 is expressed predominantly in the heart, PDK2 is relatively ubiquitously expressed in the fed state, PDK3 is found in testes, kidney and brain and PDK4 is expressed primarily in heart and skeletal muscle tissue (Bowker-Kinley *et al*. 1998).

Studies conducted on PDC isolated from heart and kidney have established that although phosphorylation occurs on three serine residues in the α-chain of E1 (designated as sites 1, 2 and 3), phosphorylation of site 1 seems to be the predominant inactivating site (for review, see Patel & Korotchkina, 2001). Subsequent studies using recombinant E1 mutant proteins revealed that PDK isoenzymes have different kinetic properties towards the three phosphorylation sites (for review Patel & Korotchkina, 2001). The four PDK isoenzymes also differ in their sensitivity to inhibition by pyruvate, with PDK4 having relatively low sensitivity to inhibition by pyruvate (Bowker-Kinley *et al*. 1998). Taken together, it has been proposed from these findings that the relative amount of the different PDK isoenzymes expressed under different physiological states may be the major determinant of total PDK activity in a given tissue and, thus, the primary factor regulating activity of the PDC (Bowker-Kinley *et al*. 1998; Sugden *et al*. 2000). Further information on structure and regulation of PDC activity has recently been presented in several reviews (Perham, 2000; Harris *et al*. 2001; Patel & Korotchkina, 2001; Reed, 2001, Roche *et al*. 2001; Sugden & Holness, 2003).

The importance of phosphorylation–dephosphorylation in regulating the activity of pyruvate dehydrogenase (PDH) was realized more than three decades ago; Hagg *et al*. (1976) stated that this interconversion seems to play a major role in regulating pyruvate oxidation by exercise and by various hormonal and nutritional states. Many experiments have been conducted over the last three decades to examine the regulation of the activity of PDH (phosphorylation state of E1). Thus, several studies have reported a decrease in PDH activity in response to fasting and alloxan–streptozotocin-induced diabetes in rat muscle (for review, see Roche *et al*. 2001; Sugden & Holness, 2003) and in response to a high-fat diet in human muscle (Putman *et al*. 1993). In addition, an exercise- or contraction-induced increase in PDH activity has been demonstrated in both rat (Hennig *et al*. 1975; Hagg *et al*. 1976; Dohm *et al*. 1986; Brozinick *et al*. 1988) and human (Ward *et al*. 1982; Putman *et al*. 1993) muscle during exercise or contractions of ≤2 h duration. Determination of PDH activity during exercise of >2 h duration has not been performed until recently (see p. 224).

Transcriptional regulation of pyruvate dehydrogenase kinase 4 in skeletal muscle in response to exercise

Wu *et al*. (1999) were the first to demonstrate regulation of PDK4 expression in skeletal muscle. Both fasting and streptozotocin-induced diabetes elicited marked increases in PDK4 mRNA and PDK4 protein in rat skeletal muscle. Several studies have confirmed these findings in rat muscle (Sugden *et al*. 2000; Holness *et al*. 2002) and similar marked up-regulation of PDK4 transcription and mRNA content has been demonstrated in human muscle in response to short-term fasting (Pilegaard *et al*. 2003*a*). Also, high-fat feeding for 28 d in rats (Holness *et al*. 2000) and for 3 d in human subjects (Peters *et al*. 2001*b*) has been shown to result in an increased expression of PDK4. The findings by Wu *et al*. (1999) led us to speculate that

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PDK4 expression might be regulated at the level of transcription in response to exercise.

The first study that investigated transcriptional regulation of PDK4 in response to exercise (Pilegaard *et al*. 2000) showed that PDK4 transcription is markedly induced by both 75 min and 4 h exhaustive exercise. PDK4 transcription and PDK4 mRNA are elevated at the end of exercise and remain high throughout 4 h of recovery after both exercise protocols. However, the levels of PDK4 transcription and PDK4 mRNA return to resting levels 24 h after exercise, revealing the transient nature of the exercise-induced PDK4 induction (Pilegaard *et al*. 2000). The finding that PDK4 transcription is elevated at the end of exercise indicates that PDK4 transcription is induced already during exercise. This possibility has been pursued by studying the pattern of PDK4 induction during prolonged exercise in more detail by taking multiple biopsies during prolonged low-intensity cycling until exhaustion (average 3·5 h; Mourtzakis *et al*. 2002). PDK4 mRNA is increased after the first 2 h of exercise, but the most marked increase occurs during recovery.

The *PDK4* gene is not only activated in response to prolonged continuous exercise; short-term high-intensity exercise, both fifteen supramaximal 1 min exercise bouts (3 min rest; Nordsborg *et al*. 2003) and five 2 min exercise bouts (3 min rest) until exhaustion (M Olesen, C Kierkegaard, B Saltin and H Pilegaard, unpublished results) have also been found to markedly increase PDK4 mRNA in untrained human skeletal muscle at 2–5 h of recovery. These findings provide evidence that PDK4 transcription is increased markedly in human skeletal muscle both during prolonged exercise and during recovery from exercise. Although protein data (PDK4 protein, PDC phosphorylation) are not yet available, the findings to date suggest that induction of the *PDK4* gene during and after exercise may trigger the phosphorylation and inactivation of the PDC complex, thus blocking entry of carbohydrates into the mitochondria for oxidation (Fig. 2(b and c)).

The potential impact of exercise intensity and duration on the fibre-type specific regulation of PDK4 in skeletal muscle has recently been investigated in rats. Low-intensity treadmill running performed for 45 min elicits a marked activation in PDK4 transcription in both red and white gastrocnemius muscle, although the response is greater in white muscle. Extending exercise duration to 3 h elicits greater activation of PDK4 transcription in white gastrocnemius muscle whereas no effect of exercise duration is evident in red muscle (Hildebrandt *et al*. 2003). Such an effect of exercise duration on PDK4 expression in rats during recovery from exercise is also in accordance with findings in human subjects (Pilegaard *et al*. 2000; C Kierkegaard, M Olesen, B Saltin and H Pilegaard, unpublished results).

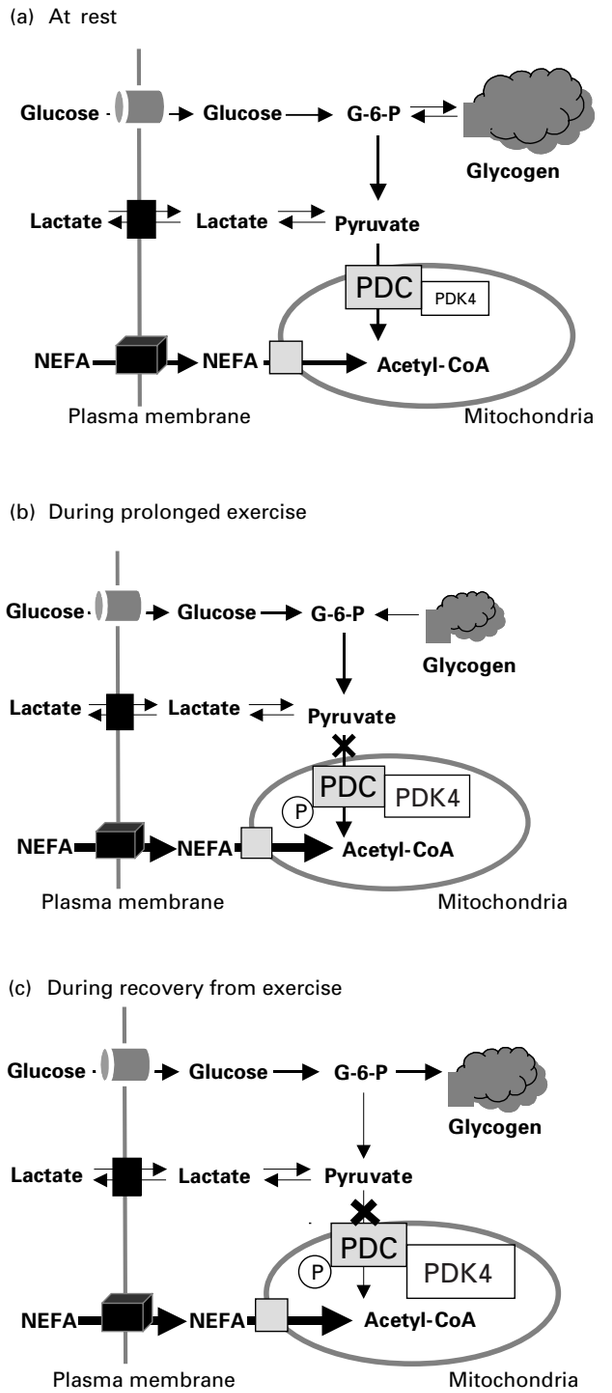

Fig. 2. The fate of glucose-6-phosphate (G-6-P) at rest (a), during prolonged exercise (b) and during recovery from exercise (c). Pyruvate dehydrogenase kinase (PDK) 4 expression is increased during prolonged exercise and during recovery from exercise causing phosphorylation and deactivation of the pyruvate dehydrogenase complex (PDC), thereby blocking the entry of carbohydrate-derived fuel into the mitochondria for oxidation.

Potential role of substrate availability in regulating pyruvate dehydrogenase kinase 4 expression

As PDK4 transcription and PDK4 mRNA content are markedly increased during and after exercise when fasting, it has been suggested that activation of the *PDK4* gene may be sensitive to substrate availability. Lowering pre-exercise muscle glycogen levels by previous exercise and intake of a low-carbohydrate high-fat diet increases PDK4 transcription and mRNA content of the low-glycogen leg

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muscle before and during prolonged exercise (Pilegaard *et al*. 2002). These findings, together with the results from a somewhat different glycogen manipulation protocol (Pilegaard *et al*. 2002), suggest that low muscle glycogen enhances the transcriptional response of the *PDK4* gene to exercise. While the amount of glycogen broken down does not seem to be a key factor *per se*, the influence of muscle glycogen on PDK4 transcription may be evident when low levels of muscle glycogen are experienced over an extended period. Whether a critical low threshold level of glycogen may be needed for enhancement of the exercise-induced PDK4 response is not known. It should be noted that other factors such as elevated plasma NEFA or changes in circulating hormones associated with the dietary manipulations may also have influenced the PDK4 expression in these studies.

The importance of substrate availability during recovery from exercise, and thus recovery of homeostasis, has been studied in subjects completing two almost identical trials with only the diet composition during recovery from exercise being different; i.e. either a high-carbohydrate diet or a low-carbohydrate high-fat diet. The diet regimens, which result in very different recovery of muscle glycogen, glucose uptake and plasma NEFA concentrations, were found to have a clear effect on PDK4 expression. After similar initial induction and return towards resting levels at 5 h of recovery in both trials, PDK4 transcription and PDK4 mRNA were again elevated late (8 h and 24 h) in recovery with the low-carbohydrate high-fat diet, but remained at resting levels from 5 h of recovery with the high-carbohydrate diet (Pilegaard *et al*. 2001). The late induction of PDK4 expression with the low-carbohydrate high-fat diet may have been elicited as a result of prolonged low muscle glycogen content or hormonal changes, or may have been related to elevated plasma NEFA levels. Whether a combination such as low glycogen and elevated NEFA is needed is still unclear and requires further investigation. However, the finding that fasting (with elevated plasma NEFA and unchanged muscle glycogen content) increases PDK4 transcription and mRNA in human muscle (Pilegaard *et al*. 2003*a*) suggests that lowering of muscle glycogen is not always necessary for the induction of PDK4 transcription.

Preliminary data (Pilegaard *et al*. 2003*b*), showing that infusion of intralipid–heparin in human subjects, resulting in markedly elevated plasma NEFA levels, enhances PDK4 transcription and mRNA content compared with saline (9 g NaCl/l) infusion suggest that NEFA may have an effect. In addition, increased PDK4 expression in skeletal muscle in response to fasting and high-fat feeding both in rat and human muscle (Wu *et al*. 1999; Hildebrandt & Neufer, 2000; Holness *et al*. 2000; Sugden *et al*. 2000; Peters *et al*. 2001*a*,*b*; Holness *et al*. 2002; Pilegaard *et al*. 2003*a*) also supports the possibility that increased availability of NEFA may play a role in regulating PDK4 expression. Together, these observations provide evidence that regulation of PDK4 transcription may be associated with muscle glycogen and/or plasma NEFA concentrations. Such regulatory mechanisms would be consistent with hypotheses that regulation of PDK4 expression plays an important role in: (1) reducing carbohydrate oxidation in muscle when whole-body carbohydrates are low; (2) promoting glycogen resynthesis, reflecting the metabolic priority given to replenishment of energy reserves during recovery from exercise (Fig. 2(b and c)).

Potential transcription factors involved in regulating the pyruvate dehydrogenase kinase 4 gene

Evidence has been presented in favour of a role for PPARα as a transcription factor involved in activating the *PDK4* gene. A marked increase in PDK4 protein and mRNA has been found in skeletal muscle when rats are fed the PPARα analogue WY14,643 for 3 d compared with control rats (Wu *et al*. 1999). Although conflicting results have been reported for PDK4 expression in PPARα-deficient mice (Wu *et al*. 2001; Holness *et al*. 2002), it seems likely that PPARα could be involved in the potential effect of NEFA on PDK4 expression, because PPARα is known to be activated by NEFA. However, results have also been obtained that indicate that an additional mechanism, other than via PPARα, may be involved in regulating PDK4 expression (Holness *et al*. 2002; Muoio *et al*. 2002).

Recent findings suggest that the transcription factor forkhead homologue in rhabdomyosarcoma (FKHR; also termed FOXO1), which belongs to the FOXO subfamily of forkhead-type transcription factors, regulates PDK4 expression in skeletal muscle. Overexpression of FKHR in C2C12 cells has been shown to increase *PDK4* gene expression, with the level of PDK4 expression being dependent on the level of FKHR expression. In addition, transfection experiments using C2C12 cells suggest that the FKHR protein may exert the effect on PDK4 expression through direct binding to the promoter region of the *PDK4* gene (Furuyama *et al*. 2003). Taken together, these findings suggest a possible role for FKHR in regulating the *PDK4* gene in skeletal muscle.

Possible role of pyruvate dehydrogenase kinase 4 expression in substrate utilization during exercise

As mentioned earlier, several studies have previously shown that PDH activity is rapidly increased during the initial 1–2 h of exercise (Hennig *et al*. 1975; Hagg *et al*. 1976; Ward *et al*. 1982; Dohm *et al*. 1986; Brozinick *et al*. 1988; Putman *et al*. 1993). The finding that PDK4 transcription and mRNA content increase during prolonged exercise, together with the known switch occurring in substrate utilization in skeletal muscle during prolonged exercise, has led to the proposal that increased PDK4 expression might have an effect on PDH activity during prolonged exercise.

The determination of PDH activity in muscle biopsies obtained every hour during prolonged low-intensity cycling until exhaustion (average 3·5 h) has demonstrated that PDH activity is increased at 1 and 2 h of exercise (reaching about 2·8-fold above resting levels) followed by a decrease towards resting levels as the exercise proceeds, with PDH activity no longer being different from rest at exhaustion (Mourtzakis *et al*. 2002). Similar results for PDH activity in human muscle during exercise have recently been

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reported (Watt *et al*. 2002). The decline in PDH activity late in exercise is in line with the possibility that increased PDK4 expression may cause an inactivation of PDC during prolonged exercise and suggests that up-regulation of PDK4 expression is an important mechanism for inhibiting PDH during prolonged exercise in order to limit the entry of glycolytic products into the mitochondria for oxidation (Fig. 2(b)). However, the decrease in PDH activity seems to occur after the switch in substrate utilization is initiated, indicating that regulatory mechanisms other than changes in PDH activity are involved during the initial hours of exercise. As acetyl-CoA carboxylase phosphorylation is markedly increased at 1 h of low-intensity exercise (Wojtaszewski *et al*. 2002), deactivation of acetyl-CoA carboxylase leading to disinhibition of carnitine palmitoyltransferase 1 and thus increased mitochondrial fatty acid uptake and oxidation may play a role in regulating the substrate choice in skeletal muscle initially during prolonged low-intensity exercise.

Concluding remarks: importance of regulating pyruvate dehydrogenase kinase 4 expression

The marked increase in PDK4 transcription and mRNA content in response to exercise (Pilegaard *et al*. 2000, 2001, 2002, 2003*b*; Mourtzakis *et al*. 2002; Nordsborg *et al*. 2003) suggests that regulation of PDK4 expression may be an important mechanism regulating the flux through the PDC complex during and after exercise. PDK4 expression also increases in skeletal muscle in response to fasting (Wu *et al*. 1999; Pilegaard *et al*. 2003*a*) and high-fat feeding (Holness *et al*. 2000; Peters *et al*. 2001*b*) in rats and human subjects and streptozotocin-induced diabetes in rats (Wu *et al*. 1999), all representing metabolic states in which carbohydrate availability is limited, as it is during and after prolonged exercise. Thus, when carbohydrate availability is low, up-regulation of PDK4 will contribute to the sparing of carbohydrates by preventing carbohydrate oxidation. The impact of substrate availability on PDK4 expression during recovery from exercise (Pilegaard *et al*. 2001) also underlines the high metabolic priority given to replenishing muscle glycogen stores and re-establishing intracellular homeostasis after exercise. The effects of manipulations in muscle glycogen and diet composition (Pilegaard *et al*. 2001, 2002) provide evidence that factors related to substrate availability are critical in the initiation of intracellular signals that lead to the regulation of PDK4 expression in skeletal muscle. The increased expression of PDK4, leading to a much larger relative contribution of PDK4 to PDK activity than the other PDK isoforms, implies that the sensitivity to pyruvate inhibition will be markedly reduced. To what extent such associated changes in inhibitor sensitivity has a functional importance is not known.

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