Different mechanisms underlie the long-term regulation of pyruvate dehydrogenase kinase (PDHK) by tri-iodothyronine in heart and liver

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Abstract Antibodies to purified recombinant PDHKII were used for ELISAs of PDHKII in mitochondrial extracts. In liver, hyperthyroidism elicited a 2.3-fold increase in PDHK activity ($P < 0.01$) which was accompanied by a significant 1.5-fold ($P < 0.001$) increase in the amount of mitochondrial immunoreactive PDHKII. In contrast, despite a stable 2.0-fold increase in cardiac PDHK activity ($P < 0.001$), the amount of mitochondrial immunoreactive PDHKII in heart was unaffected by hyperthyroidism. The mechanisms for long-term regulation of PDHK activity by thyroid hormones therefore differ fundamentally between heart and liver.

Key words: ELISA; PDHKII; Pyruvate dehydrogenase

1. Introduction

The present study examines the molecular basis for the increases in cardiac and hepatic pyruvate dehydrogenase kinase (PDHK) activities observed in response to hyperthyroidism in vivo using ELISAs for recombinant PDHKII, the major PDHK isoform in rat heart and essentially the sole PDHKII isoform in rat liver [1]. The aim was to determine whether the increased PDHK activities elicited by hyperthyroidism in vivo are due to changes in enzyme concentration and/or specific activity.

PDHK catalyses the phosphorylation of the α-subunit of the pyruvate dehydrogenase (PDH) component of the pyruvate dehydrogenase complex (PDHC), leading to its inactivation (reviewed in [2]). Increasing mitochondrial [acetyl-CoA]/[CoA] and [NADH]/[NAD^+] ratios activate PDHK, whereas increasing pyruvate concentrations suppress its activity [3]. It has been proposed previously that the long-term increase in PDHK activity in liver observed in response to prolonged starvation is a consequence of an increase in PDHK specific activity rather than increased PDHK protein (reviewed in [4]; see also [5]). In experiments in tissue culture with hepatocytes and cardiac myocytes, fatty acids and cAMP have been shown to be possible mediators of the effects of starvation to increase PDH kinase activity [6–9].

Hyperthyroidism is associated with increased rates of fatty acid oxidation in these tissues [10,11]. The responses of cardiac and hepatic PDHK to hyperthyroidism occur over a timescale comparable with those evoked by starvation (2–3 days) [12,13], and may be important in facilitating the increased Cori recycling characteristic of hyperthyroid state [10,14,15]. Studies with cultured cardiac myocytes [8] and hepatocytes [12] have demonstrated direct tissue effects of tri-iodothyronine (T3) to enhance PDHK activity. These effects are opposed by the further addition of insulin to the culture medium [8] and, in hepatocytes, by inhibition of mitochondrial fatty acid oxidation [12]. The similar characteristics of the long-term effects of starvation and hyperthyroidism to evoke stable increases in heart and liver PDHK activities initially suggested that they might share a common underlying mechanism, but subsequent evidence indicates that the effects of T3 is different to that of starvation. For example, additivity between the effects of starvation and hyperthyroidism to increase cardiac PDHK activity would imply that separate mechanisms may be involved [8]. Also, in liver, although increases in PDHK in response to hyperthyroidism are observed in the presence of a modest increase in hepatic cAMP concentrations, dose response studies demonstrated a dissociation between changes in PDHK activities and cAMP concentrations [12]. It was nevertheless possible to detect a positive correlation between effects of T3 administration to elevate fatty acid supply and hepatic PDHK activity [12].

2. Materials and methods

Biochemicals and chemicals were from Boehringer Corp. or from Sigma Chemical Corp., Poole, Dorset, UK. Recombinant PDHKII was kindly provided by Zeneca Pharmaceuticals, UK. Female Wistar rats were purchased from Charles River Ltd., Margate, Kent, UK.

Female Albino Wistar rats were maintained on a 12 h light/12 h dark cycle (light from 10.00 h) and sampled in the absorptive state at the end of the dark phase. Rats were permitted free access to standard rodent diet (8% fat, 72% carbohydrate and 20% protein, by calories) and to water.

Active PDHC (PDHa) activities were assayed spectrophotometrically by coupling to arylamine acetyltransferase. Total PDHC was assayed as active complex after incubation of mitochondria for 10 min in the absence of respiratory substrate. PDHK activities were assayed at pH 7.0 in extracts of heart mitochondria by the rate of ATP-dependent inactivation of fully activated PDHC and computed as apparent pseudo first order rate constants for ATP-dependent PDHa inactivation [16]. Details of these methods are provided in [5,9,16].

Antibodies were raised in New Zealand White rabbits to purified recombinant PDHKII (provided by Zeneca Pharmaceuticals) adjudged to be 49% pure on densitometric scanning of an SDS-PAGE gel stained with coomassie blue. The priming dose was 10 μg of protein in Freund's complete adjuvant given subcutaneously at four dorsal sites. After 6 weeks, boosting injections (10 μg) in Freund's incomplete adjuvant were given at 4-weekly intervals. Blood was removed from an ear vein at 10 days after boosts. The serum obtained was screened for antibodies by Western blotting from SDS/PAGE using preimmune serum as control. The antiserum was specific to PDHKII with negligible cross-reaction to PDKI as assessed by Western blots (results not shown). ELISA assays were performed using clarified extracts of liver or heart mitochondria as described in [17].

Results are means ± standard error (S.E.M.) for the numbers of observations indicated. Statistical analyses were made with StatView (Abacus Concepts, Inc., Berkeley, CA). Statistical significance of dif-

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ferences between groups was assessed by Student's unpaired *t*-test. Curve-fitting was carried out using *Fig P* software.

3. Results

3.1. PDHK activities in heart and liver mitochondria after T3 treatment in vivo

PDHK activity measured in extracts of liver mitochondria was increased from by 2.3-fold, from 1.83 ± 0.28 min^{-1} ($n = 10$) to 3.82 ± 0.45 min^{-1} ($n = 10$) ($P < 0.01$) after treatment with T3 for 3 days. PDHK activity measured in extracts of cardiac mitochondria was increased from 1.01 ± 0.13 min^{-1} ($n = 7$) to 2.05 ± 0.22 min^{-1} ($n = 7$) ($P < 0.001$) after treatment with T3 for 3 days. As reported previously [8,12,18], total PDHC activities in heart and liver mitochondria were unaffected by hyperthyroidism (results not shown).

3.2. ELISAs of PDHKII in mitochondrial extracts

We examined whether the PDHK activity increases in liver and heart were accompanied by increases in PDHKII enzyme concentrations. The results for ELISAs of PDHKII in clarified extracts of liver mitochondria from euthyroid and hyperthyroid rats are shown in Fig. 1a. In these experiments, we compared the two groups (hyperthyroid versus euthyroid) directly through side-by-side comparison, in the same well block and over the same range of PDHC activities (from 0.03 to 5 m-units/well). The amount of immunoreactive PDHKII in mitochondrial extracts from livers of fed hyperthyroid rats was reproducibly and consistently increased over the entire range of PDHC activities. Individual points for the ELISA are shown in the inset to Fig. 1b. Immunoreactive PDHKII

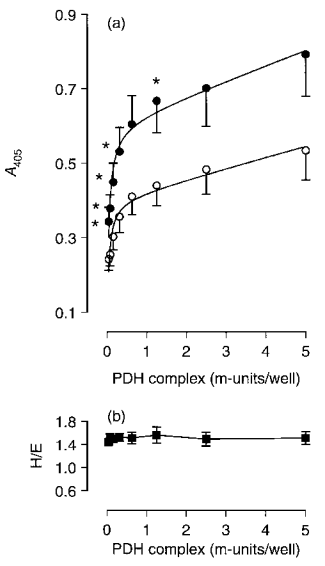

Fig. 1. ELISA of PDHKII in extracts of mitochondria from livers of euthyroid (E) and hyperthyroid (H) rats. Mitochondria from euthyroid rats (open symbols) and hyperthyroid rats (closed symbols) were extracted by freezing and thawing (three times) in mitochondrial extraction buffer. The extracts were clarified by centrifugation and ELISAs performed with pairs of extracts as described in [15]. Data are shown in (a) and ratios of hyperthyroid/euthyroid (H/E; means ± S.E.M.) for individual points in the ELISA in (b). Results are means ± S.E.M. for ELISAs of four separate mitochondrial preparations (each with four wells for each PDH complex activity). Statistically significant differences between euthyroid and hyperthyroid rats are indicated by: $*P < 0.05$.

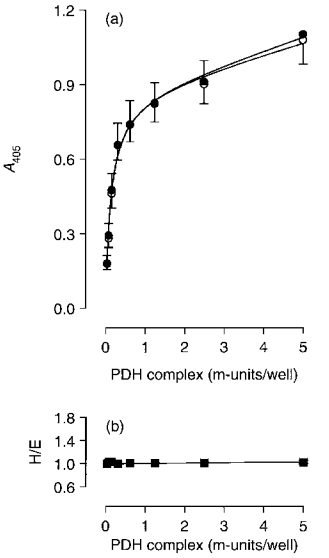

Fig. 2. ELISA of PDHKII in extracts of mitochondria prepared from hearts of euthyroid (E) and hyperthyroid (H) rats. Mitochondria from euthyroid rats (open symbols) and hyperthyroid rats (closed symbols) were extracted by freezing and thawing (three times) in mitochondrial extraction buffer. The extracts were clarified by centrifugation and ELISAs performed with pairs of extracts as described in [15]. Data are shown in (a) and ratios of hyperthyroid/euthyroid (H/E; means ± S.E.M.) for individual points in the ELISA in (b). Results are means ± S.E.M. for ELISAs of four separate mitochondrial preparations (each with four wells for each PDH complex activity).

protein was 1.3- to 1.8-fold greater in extracts from hyperthyroid (H) rats than that in extracts from euthyroid (E) rats with a mean ratio of activities at individual points in the ELISA of 1.503 ± 0.018 ($n = 40$).

Fig. 2a shows the results for ELISA assay of PDHKII in clarified extracts of heart mitochondria from euthyroid and hyperthyroid rats. As with the liver, the assay was again conducted over a range of PDH activities (from 0.03 to 5 m-units/well). The amount of immunoreactive PDHKII in mitochondrial extracts from hearts of hyperthyroid rats (H) was ≈ similar to that in extracts from the euthyroid rats (E) with no significant difference between the individual values (see the inset to Fig. 2b).

4. Discussion

PDHK comprises one component of the regulatory phosphorylation cycle of mammalian PDHC. It comprises 2 dissimilar α- and β-subunits. The α-subunit is the catalytic subunit. PDHKα is partly tightly bound to PDHC, and is partly free in the mitochondrial matrix. The more loosely bound PDHK is identical with the so-called PDHK activator protein, KAP [19,20]. Prolonged starvation causes an increase in PDHK activity and it was concluded from studies employing polyclonal antibodies raised to PDHKα purified from livers of 48 h starved rats that no significant differences in the concentration of this protein existed between livers from fed and starved rats [17]. Culture of cardiac myocytes or hepatocytes prepared from fed rats with a fatty acid (*n*-octanoate or palmitate) and dibutyryl cyclic AMP (Bt_2cAMP) for 24 h mimics the long-term effect of starvation to increase PDHK activity [6,7]. Hyperthyroidism also leads to stable increases in PDHK

[8,12] and the responses of cardiac and hepatic PDHK to hyperthyroidism occur over a similar timescale [8,12]. Also, studies with cultured cardiac myocytes [8] and hepatocytes [12] have shown direct effects of tri-iodothyronine (T3) to enhance PDHK activity.

Multiple isoenzymic forms of PDHK have been identified [1,21]. PDHKI and PDHKII have been detected in rat tissues. In tissues tested thus far, the expression of PDHKII mRNA is higher than that of either PDHKI (rat, human) or PDHKIII (human) [1,21]. PDHKI and PDHKII are both present in rat heart, but PDHKII is the only isoform of PDHK detected in significant quantity in rat liver to date [1]. The present study examined whether the concentration of a specific isoform of PDHK, PDHKII ($M_r = 45$ kDa), was altered by hyperthyroidism in the fed state, either in heart or in liver.

Experiments in vivo have demonstrated that the long-term response of PDHK activity to starvation is blocked by cycloheximide [16], suggestive of an effect at the level of transcription and/or translation. In addition, cycloheximide almost totally inhibits the 3-fold increase in PDHK activity induced by Bt_2cAMP in cultured hepatocytes [17]. Here we demonstrate that the long-term action of hyperthyroidism in vivo to increase hepatic PDHK activity is associated with a stable increase in the concentration of hepatic PDHKII. Further studies will resolve whether the long-term effect of hyperthyroidism to increase hepatic PDHKII concentration is exerted at the level of transcription; however, it is known that the T3 receptor is a member of the steroid/thyroid/retinoid superfamily of nuclear hormone receptors and T3 is capable of inducing gene expression via a T3 response element.

It would be predicted that, in the absence of any change in the specific activity of hepatic PDHK in hyperthyroidism, the increase in PDHK activity would be paralleled by an increase in PDHKII concentration of similar magnitude. The mean fold increase in PDHKII activity elicited by hyperthyroidism (2.3 ± 0.3) exceeds the mean fold increase in PDHKII protein (1.5 ± 0.1). Effects of T3 to increase PDHK in cultured hepatocytes can be reversed by inhibition of mitochondrial fatty acid oxidation [12], but the effect of fatty acids to increase PDHK activity in cultured hepatocytes is not blocked significantly by cycloheximide [17]. Taken together, the results suggest that post-translational modification secondary to enhanced fatty acid oxidation may, in conjunction with increased PDHKII expression, participate in achieving the overall enhancement of hepatic PDHK activity elicited by hyperthyroidism. Thus, a dual mechanism of enhanced specific activity in association with increased PDHKII protein operates in liver to increase PDHK activity in response to hyperthyroidism.

The current study reveals that T3 administration increases cardiac PDHK activity, without any increase in the tissue concentration of PDHKII. The results of the ELISA for PDHII exclude a change in PDHKII concentration as the mechanism by which cardiac PDHK activity is stably enhanced in hyperthyroidism. However, in contrast with rat liver (which contains PDHKII), rat heart contains PDHKI ($M_r = 48$ kDa), which exhibits approx. 70% sequence homology with PDHKII, in significant amounts. Our data do not exclude the possibility of a change in the cardiac concentration of PDHKI or a further PDHK isoform. Alternatively, the effect of hyperthyroidism to increase cardiac PDHK activity may be achieved through post-translational modification of PDHKI/II, with a concomitant increase in PDHK specific activity.

In summary, our data demonstrate that the effect of hyperthyroidism to increase hepatic PDHK activity is associated with a specific increase in PDHKII protein, although a parallel increase in PDHK specific activity is not excluded. In addition, PDHK may be added to the cohort of many gene products whose expression in liver is regulated by changes in thyroid status. The study also clearly demonstrates that hyperthyroidism increases the activity of cardiac PDHK without any change in the tissue concentration of PDHKII (one of the major isoforms of PDHK in rat heart), as assessed by ELISAs of PDHKII in mitochondrial extracts. It can therefore be concluded that the long-term regulation of PDHK differs between these important tissues, and that results and conclusions obtained with one tissue preparation cannot necessarily be extrapolated to another.

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