Activation of the cardia mTOR/p70S6K pathway by leucine requires PDK1 and correlates with PRAS40 phosphorylation

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Activation of the cardiac mTOR/p70S6K pathway by leucine requires PDK1 and correlates with PRAS40 phosphorylation. The signaling pathway mediating the amino acid-dependent activation of mTOR/p70S6K is not yet entirely understood. In contrast, the signaling leading to its activation by insulin has been almost fully elucidated and involves the canonical association of phosphatidylinositol 3-kinase (PI3K), phosphoinositide-dependent protein kinase-1 (PDK1), and protein kinase B (PKB/Akt). The signaling involved in leucine effect, although known to implicate a PI3K mechanism independent of PKB/Akt, is more poorly understood. In this study, we investigated whether PDK1 could also participate in the events leading to mTOR/p70S6K activation in response to leucine in the heart. In wild-type hearts, both leucine and insulin increased p70S6K activity whereas, in contrast to insulin, leucine was unable to activate PKB/Akt. The changes in p70S6K activity induced by insulin and leucine correlated with changes in phosphorylation of Thr389, mTOR phosphorylation site on p70S6K, and of Ser2448 on mTOR, both related to mTOR activity. Leucine also triggered phosphorylation of the proline-rich Akt/PKB substrate of 40 kDa (PRAS40) (50, 51). Once phosphorylation on Thr229 in the activation loop of p70S6K, it promotes the association of mTOR with the target of rapamycin complex 1 (TORC1) (26, 27). The activation of mTOR/p70S6K is not yet entirely understood. By Thr389 phosphorylation, mTOR is the determining step of p70S6K activation (16, 27, 40, 52).

The molecular mechanism by which amino acids regulate p70S6K also involves mTOR (13, 22, 41). Moreover, although amino acid-dependent activation of mTOR/p70S6K is prevented by PDK1 inhibitor, it does not involve PKB/Akt activation (19, 30, 43). It seems to be controlled by a class 3 PI3K (20, 21). However, the intermediates between this PI3K and mTOR/p70S6K remain to be discovered. In the present work, we studied the implications of PDK1 in the leucine-induced activation of p70S6K. This hypothesis is based on the following arguments. First, PDK1 is known to be regulated by a PI3K-mediated production of phosphoinositides, and a PI3K is involved in the amino acid effect. Second, PKB/Akt is not the sole protein kinase targeted by PDK1. Indeed, PDK1 possesses several other protein kinases from the AGC family as substrates (38). Third, the skeletal and cardiac muscle specific PDK1 knockout (PDK1−/−) mouse is myogenic cells (15). In heart, p70S6K phosphorylation has been demonstrated after oral administration of leucine (34), and glutamine has been reported to activate the mTOR/p70S6K pathway in neonatal cardiomyocytes (53).

The signaling pathway mediating the amino acid-dependent activation of mTOR/p70S6K is not yet entirely understood. By contrast, the signaling leading to its activation by insulin has been almost fully elucidated and involves the canonical association of phosphatidylinositol 3-kinase (PI3K), phosphoinositide-dependent protein kinase-1 (PDK1), and protein kinase B (PKB/Akt) (2, 8, 13). Insulin acts via the recruitment and the stimulation of the class I PI3K at the plasma membrane. This lipid kinase produces phosphatidylinositol 3,4,5-triphosphate, which binds to the pleckstrin homology domain of both PKD1 and PKB/Akt (38). This leads to the PDK1-induced activation of PKB/Akt by phosphorylation on Thr389. Once activated, PKB/Akt induces mTOR activation via two concomitant mechanisms. First, PKB/Akt phosphorylates and inactivates the tuberous sclerosis complex 2 (TSC2), the negative regulator of the G protein Rheb (25, 44). The resulting activated form of Rheb, in turn, allows mTOR activation. The second mechanism implicates the proline-rich Akt/PKB substrate of 40 kDa (PRAS40) (50, 51). Once phosphorylated by PKB/Akt, PRAS40 binds to 14-3-3, thereby relieving the PRAS40-induced inhibition of mTOR and eventually allowing its action on p70S6K. The insulin-induced activation of mTOR by PKB/Akt correlates with its phosphorylation of Ser2448 (2). Once activated, mTOR phosphorylates p70S6K on Thr389. This phosphorylation induces a conformational change that exposes Thr229 in the activation loop of p70S6K and allows its phosphorylation by PDK1, resulting in p70S6K activation (1, 46). Phosphorylation of Thr389 by mTOR is the determining step of p70S6K activation (16, 27, 40, 52).

The molecular mechanism by which amino acids regulate p70S6K also involves mTOR (13, 22, 41). Moreover, although amino acid-dependent activation of mTOR/p70S6K is prevented by PI3K inhibitors, it does not involve PKB/Akt activation (19, 30, 43). It seems to be controlled by a class 3 PI3K (20, 21). However, the intermediates between this PI3K and mTOR/p70S6K remain to be discovered. In the present work, we studied the implication of PDK1 in the leucine-induced activation of p70S6K. This hypothesis is based on the following arguments. First, PDK1 is known to be regulated by a PI3K-mediated production of phosphoinositides, and a PI3K is involved in the amino acid effect. Second, PKB/Akt is not the sole protein kinase targeted by PDK1. Indeed, PDK1 possesses several other protein kinases from the AGC family as substrates (38). Third, the skeletal and cardiac muscle specific PDK1 knockout (PDK1−/−) mouse is myogenic cells (15). In heart, p70S6K phosphorylation has been demonstrated after oral administration of leucine (34), and glutamine has been reported to activate the mTOR/p70S6K pathway in neonatal cardiomyocytes (53).
characterized by the reduction of cardiomyocyte size and dilated cardiomyopathy (37). This cardiomyopathic phenotype is more severe than that usually found in cardiac-specific knockout of the other proximal elements of insulin signaling (37), signifying that PDK1 should play an important role in pathways regulating cell size and growth other than those involving insulin and PKB/Akt. So, the aims of the present work were 1) to define the effects of both leucine and glutamine on the mTOR/p70S6K pathway in perfused heart and 2) to evaluate the role of PDK1 in this amino acid action by using two different genetic models, the skeletal and cardiac muscle-specific PDK1 L155E (37) and the PDK1 L155E knock-in (KI) (3) mice. In the first model, the insulin-induced activation of the PKB/Akt/mTOR/p70S6K pathway is abrogated (37). In the PDK1 L155E KI model, the PDK1-dependent activation of PKB/Akt by insulin persists, whereas activation of p70S6K by phosphorylation on Thr229 is abolished (9, 12).

MATERIALS AND METHODS

Materials. Radiochemicals and protein A-Sepharose were obtained from Amersham Biosciences. Insulin, L-glutamine, and L-leucine were from Novo Nordisk, Calbiochem, and Fluka, respectively. Synthetic peptides for PKB/Akt (RPRAATF) and p70S6K (KKRNRTLSVA) (KRNRTLSVA) were from the indicated sources. The Anti-PRAS40, and mTOR. The appropriate secondary antibody conjugated to peroxidase was from Amersham Biosciences. Insulin, L-glutamine, and L-leucine were from Novo Nordisk, Calbiochem, and Fluka, respectively. Synthetic peptides for PKB/Akt (RPRAATF) and p70S6K (KKRNRTLSVA) (KRNRTLSVA) were from the indicated sources. The Anti-PRAS40, and mTOR. The appropriate secondary antibody conjugated to peroxidase was from Amersham Biosciences. Insulin, L-glutamine, and L-leucine were from Novo Nordisk, Calbiochem, and Fluka, respectively. Synthetic peptides for PKB/Akt (RPRAATF) and p70S6K (KKRNRTLSVA) (KRNRTLSVA) were from the indicated sources. The Anti-PRAS40, and mTOR. The appropriate secondary antibody conjugated to peroxidase were from Sigma and BD Biosciences, respectively. Unless otherwise stated, the sources of all other materials are given in Refs. 7 and 55.

Animals. This study was approved by the Animal Research Committee of the Université catholique de Louvain and conformed to the American Heart Association Guidelines for Use of Animals in Research. Adult NMRI mice (12 wk old) were used for the wild-type studies. Skeletal and heart muscle-specific PDK1 L155E mice and their control littermates (PDK1+/−), both in back-crossed C57BL/6J strain, have been previously described (37). The PDK1 L155E−/− mice developed dilated cardiomyopathy when they reach 8 wk. To prevent any side effects resulting from this cardiomyopathy, we used young (4- to 5-wk-old) PDK1−/− mice and their control littermates (also called PDK1+/−) here before any visibly disturbed phenotype could be detected [studied by echocardiography as described (55)]. On the other hand, skeletal and heart muscle-specific PDK1 L155E KI mice (both in back-crossed C57BL/6J strain), which were viable and displayed no particular phenotype (3), were studied in adult stage when they were 12 wk old.

Perfusion protocol. The hearts from overnight-fasted mice anesthetized with pentobarbital sodium (1.98 mg/kg body wt ip) were excised and perfused retrogradely at 37°C at a constant pressure of 70 mmHg with a Krebs-Henseleit buffer containing 1.5 mM CaCl2, 11 mM glucose, and 1.1 mM mannitol in equilibrium with a 95% O2-5% CO2 gas phase (55). Preliminary experiments showed that the maximal effects of leucine or glutamine were observed in the 5–10 mM concentration range (see supplemental data; supplemental materials are found with the online version of this paper) and after 15 min of perfusion (data not shown). Therefore, the following protocol was adopted. After a 15-min equilibration period, insulin (100 nM), L-glutamine, or L-leucine (10 mM), alone or in combination, were added to the perfusate, and the hearts were further perfused for 15 min. At the end of the procedure, the hearts were immediately freeze-clamped in liquid nitrogen and stored at −80°C. Frozen hearts were homogenized at 4°C in 9 vol (vol/wt) of homogenization buffer that contained phosphatases and protease inhibitors as described (55).

After centrifugation (10,000 g, 30 min, 4°C), the supernatants were used immediately or stored at −80°C.

Study of leucine effect in vivo. C57BL/6J male mice were fasted overnight and then randomly assigned for treatment. Anesthetized mice (ketamine-xylazine, 80 mg · 10 mg−1 · kg−1) were injected with saline solution or leucine (10 mM) into the inferior vena cava. Hearts were collected 10 min after injection and freeze-clamped in liquid nitrogen to be stored at −80°C.

Enzyme assays and immunoblot analysis. Protein content of supernatants was determined by Bradford’s method with bovine serum albumin used as a standard. PKB/Akt and p70S6K activities were assayed after immunoprecipitation with the anti-PH domain of PKB/Akt (6, 30) and the anti-total p70S6K antibody, as previously described (30, 31). Briefly, the immunoprecipitates were incubated in the appropriate buffer (6, 31) with 0.2 mM peptide substrate and 0.1 mM [γ-32P]ATP-Mg (specific radioactivity 1,000 cpm/pmol) for 20 min at 30°C with continuous agitation. One unit of p70S6K or PKB/Akt activity corresponds to one nanomole of product formed per minute under the assay conditions. For immunoblotting, heart homogenates (30–50 μg) were subjected to SDS-PAGE and transferred onto PVDF membrane. Blots were probed with the appropriate antibodies to assess phosphorylation state and protein level of p70S6K, PKB/Akt, PRAS40, and mTOR. The appropriate secondary antibody conjugated to peroxidase and the BM chemiluminescence blotting system (Roche) were used for detection. The bands were visualized by chemiluminescence (Roche). Band intensities were quantified by scanning and processing with the program ImageJ (v. 1.33 for Mac OS X). The quantification given by antiphospho antibodies was normalized using an internal loading control (eEF2).

[14C]phenylalanine incorporation into proteins. Rat neonatal cardiomyocytes were prepared as described in the supplemental data. Cells were treated with or without L-leucine (10 mM) and [14C]phenylalanine (PerkinElmer) at 3 μCi/ml and incubated for 24 h at 37°C. Cells were then washed twice with ice-cold PBS and lysed in a cold buffer as described in the supplemental data. Proteins of the supernatants were precipitated with TCA (10%, vol/vol) for 20 min and washed with 100 mM NaOH. The pellet, containing proteins, was dissolved with formic acid, and incorporated radioactivity was detected in a liquid scintillation counter.

Statistical analysis. The results are expressed as means ± SE. Unless otherwise stated, statistical significance was calculated using a one-way (when one variable was studied) or two-way (when the two independent variables genotype and treatments were studied) analysis of variance using the Bonferroni post hoc test. The significance threshold was set at P < 0.05.

RESULTS

Leucine, but not glutamine, activated p70S6K. We first investigated the individual and combined effects of leucine and glutamine compared with insulin on p70S6K activity in perfused adult mouse hearts. The concentration of insulin (100 nM) and amino acid (10 mM) used are known to give maximal effect in various models (5, 7, 15, 30, 53, 54). Both insulin and leucine, alone or in combination, activated p70S6K (Fig. 1A). In contrast to leucine, glutamine decreased p70S6K activity compared with untreated hearts and inhibited the insulin-induced p70S6K activation. Glutamine did not modify the stimulatory effect of leucine. We next measured the phosphorylation state of p70S6K on Thr389, the mTOR phosphorylation site (Fig. 1B). Comparison of Fig. 1, A and B, reveals that Thr389 phosphorylation state paralleled p70S6K activity. The effect of leucine was also evaluated in vivo. Injection of leucine in mice induced a fourfold increase in cardiac p70S6K activity (Fig. 2A). This activation correlated well with the increase in p70S6K phos-
The effects of insulin or amino acids on PKB/Akt phosphorylation were quantified by scanning. Means \( \pm \) SE from 5–11 hearts per condition are shown. \( a \) vs. untreated hearts, \( b \) vs. insulin-treated hearts. C, control; a.u., arbitrary units.

Neither glutamine nor leucine increased PKB/Akt activity and phosphorylation. As already demonstrated in different tissues including liver (30) and muscle (19), PKB/Akt is activated by insulin but not by amino acids. We confirmed in perfused mouse hearts that insulin activated PKB/Akt and that neither leucine nor glutamine, alone or in combination, modified PKB/Akt activity (Fig. 3A). Both amino acids had no significant effect on the insulin-induced PKB/Akt activation. The effects of insulin or amino acids on PKB/Akt phosphorylation on both Thr\(^{389} \) and Ser\(^{473} \) (Fig. 3B) paralleled their effects on PKB/Akt activity. These results were in line with preceding reports (19, 30, 43), which indicated that amino acid signaling to p70S6K does not involve PKB/Akt.

Leucine, but not glutamine, increased mTOR phosphorylation. The insulin-induced activation of mTOR by PKB/Akt is known to correlate with mTOR phosphorylation on Ser\(^{2448} \). As shown in Fig. 4A, leucine, similarly to insulin, enhanced mTOR phosphorylation on the same site. These two stimulating agents together did not further increase this phosphorylation. The relatively small modification in mTOR phosphorylation state compared with large changes in p70S6K activation and phosphorylation (for both insulin and leucine) is in agreement with previous observations made in several other tissues (10, 23, 25, 48). On the other hand, glutamine inhibited Ser\(^{2448} \) phosphorylation of mTOR compared with untreated hearts. Furthermore, glutamine significantly decreased the insulin-induced mTOR phosphorylation (Fig. 4B). Therefore, the effects of insulin and amino acids on mTOR phosphorylation correlated with those found on p70S6K activity, revealing a clear association between mTOR and p70S6K in all conditions. Moreover, the correlation between mTOR phosphorylation and p70S6K phosphorylation on Thr\(^{389} \), its mTOR-dependent site, indicates that both phosphorylation sites are good indicators of mTOR activity.

Effects of leucine on both p70S6K and mTOR were abolished in PDK1\(^{−/−} \) mouse hearts. It is known that PDK1 plays a crucial role in the insulin-signaling pathway involved in the activation of PKB/Akt. Knowing that PDK1 mediates the activation of various protein kinases other than PKB/Akt, we decided to study its role in the leucine-induced activation of mTOR/p70S6K. This was first assessed by comparing PDK1\(^{+/+} \) and PDK1\(^{−/−} \) mice. As expected (37), insulin was unable to increase PKB/Akt activity in perfused heart of PDK1\(^{−/−} \) mice (PDK1\(^{+/+} \): 1.34 \pm 0.23 and 4.47 \pm 0.78 mU/mg protein for untreated and insulin-treated hearts, respectively, \( P < 0.05; \) PDK1\(^{−/−} \): 0.63 \pm 0.11 and 0.60 \pm 0.13 mU/mg protein, respectively, \( P > 0.05, n = 3, \) unpaired t-test). Both insulin and leucine treatment increased p70S6K activity in PDK1\(^{+/+} \) mouse hearts but failed to do so in PDK1\(^{−/−} \) mouse hearts (Fig. 5A). This result was rather expected, because PDK1 is known to phosphorylate p70S6K on Thr\(^{389} \). This phosphor-
findings, leucine required PDK1 not only for the ultimate step of activation of p70S6K but also for its mTOR-dependent phosphorylation on Thr389 and, more upstream in the pathway, for the phosphorylation of mTOR on Ser2448.

Action of leucine and insulin on p70S6K activity and mTOR phosphorylation was differentially altered in PDK1 L155E KI mouse hearts. PDK1 uses different molecular mechanisms to modulate the activity of its targets (38). PKB/Akt is activated by PDK1 via their combined recruitment to the plasma membrane by phosphatidylinositol 3,4,5-trisphosphates. On the other hand, PDK1 recognizes and binds to p70S6K when phosphorylated on Thr389 by mTOR (38). The region of the catalytic domain of PDK1 involved in the recognition of the phosphorylated Thr389 form of p70S6K is called the PRK2-interacting fragment (PIF)-binding pocket (9, 12). In this domain, Leu155 plays a critical role. Its mutation in glutamate (L155E) prevents the association of PDK1 with p70S6K, the phosphorylation of p70S6K on Thr229 and, so, its activation under insulin treatment (9, 12). By contrast, L155E mutation still allows insulin-induced PKB/Akt activation (3, 9). We decided to use PDK1 L155E KI mice to further study the interaction between PDK1 and the mTOR/p70S6K pathway in response to leucine. We first verified PKB/Akt activation in the hearts of PDK1 L155E KI mice and of their control littermates under insulin perfusion. As expected, insulin was able to activate PKB/Akt in both strains (untreated hearts: 0.46 ± 0.07 and 0.29 ± 0.10 mU/mg protein; insulin-treated hearts: 1.68 ± 0.38 and 1.20 ± 0.22 mU/mg protein for PDK1+/+ and PDK1 L155E KI mice, respectively, n = 5, P < 0.05 vs. untreated hearts, unpaired t-test). In contrast, p70S6K activity was almost completely abrogated in heart of PDK1 L155E KI mice in all treatments (Fig. 7A). Although p70S6K activity was absent in these mouse hearts, PKB/Akt-dependent phosphorylation of p70S6K on Thr389 was still present under insulin treatment (Fig. 7B). This phosphorylation was, however, less pronounced than in control PDK1+/+ mice, in agreement with a previous report, which explained this phenomenon by the fact that the phosphorylation of Thr389 could facilitate the phosphorylation of Thr389 (12). In contrast to what happened in insulin-treated hearts, leucine was unable to induce any substantial p70S6K phosphorylation on Thr389 in the heart of PDK1 L155E KI mice (Fig. 7B). Thr389 phosphorylation was in line with the phosphorylation of mTOR on Ser2448 (Fig. 7C). Indeed, insulin, but not leucine, normally increased mTOR phosphorylation in the hearts of PDK1 L155E KI mice. These results demonstrated the crucial role of

Fig. 4. Insulin and amino acid effects on mammalian target of rapamycin (mTOR) phosphorylation in WT mouse hearts. WT mouse hearts were treated as described in Fig. 1. A: PKB/Akt activity was determined in homogenates (100 μg of proteins) after immunoprecipitation. B: heart homogenates (30 μg of protein) were subjected to 10% SDS-PAGE and immunoblotted with anti-phospho-Ser473, anti-phospho-Thr308, or anti-total PKB/Akt antibody. Means ± SE from 5–10 hearts per condition are shown. aP < 0.05 vs. untreated hearts, bP < 0.05 vs. insulin-treated hearts.
PDK1 in leucine action on mTOR/p70S6K. They also showed that PDK1 acts differently in leucine and insulin signaling. Action of leucine on mTOR/p70S6K correlated with PRAS40 phosphorylation. It has been shown recently that PRAS40 is a critical link between PKB/Akt and mTOR/p70S6K downstream insulin (50, 51). We first verified this assessment in PDK1 genetic models (Fig. 8, A and B). As expected, the insulin-induced PRAS40 phosphorylation was fully preserved in L155E KI mice, where PKB/Akt stimulation is preserved, whereas it was greatly reduced in PDK1/H11002 mouse hearts. The still remaining PRAS40 phosphorylation in these PDK1/H11002 mice could result from a PDK1/PKB-independent mechanism or from the noncardiomyocytic cells that are present in the cardiac preparation and still express PDK1. On the other hand, little is known about the putative implication of PRAS40 in the leucine effect. In perfused wild-type mouse hearts, leucine treatment induced the phosphorylation of PRAS40 on Thr246, the site known to be the target of PKB/Akt in response to insulin (Fig. 8). This phosphorylation disappeared in both PDK1/H11002 (Fig. 8C) and PDK1 L155E KI (Fig. 8D) mouse hearts. These results revealed that a protein kinase different from PKB/Akt should be involved in the leucine-mediated phosphorylation of PRAS40 and that this protein kinase should be controlled by PDK1 in a PIF-binding pocket-dependent manner. p70S6K is one of the protein kinases regulated by PDK1 in such a way. For that reason, we decided to eliminate any possibilities of the presence of a positive feedback loop in which p70S6K, itself, would be the kinase responsible for PRAS40 phosphorylation. So, we measured the phosphorylation state of PRAS40 from perfused wild-type hearts treated with leucine or insulin in the presence or not of rapamycin, the specific mTOR/p70S6K pathway (Fig. 9). Whereas the insulin- and leucine-dependent phosphorylations of p70S6K were completely abrogated by rapamycin, PRAS40 phosphorylation was maintained, indicating that p70S6K is not the kinase involved in the phosphorylation of PRAS40 (Fig. 9).

**DISCUSSION**

The heart is in a dynamic recycling state characterized by a continuous degradation and synthesis of cardiac proteins, including contractile components. It is now accepted that not only classical energy-providing substrates but also amino acids may be considered as anabolic regulators of this recycling. Because of their ability to regulate protein turnover and, so, cardiac function, amino acids are endowed with potential therapeutic capacities.
Treatment with glutamine or leucine is known to improve myocardial function and metabolism during cardiac ischemic injury or in diabetic heart via the stimulation of cardioprotective pathways (26, 36, 42). These amino acids have also been shown to be potentially protective against burn-induced cardiac alterations (32) and cardiac atrophy (17). Finally, defect in leucine response is involved in alcohol-induced alterations in the heart (33). Amino acid-induced regulation of protein translation could be related to some of their protective effects, and this is the main reason why we were interested in studying the molecular mechanism regulating the mTOR/p70S6K pathway downstream of leucine and glutamine in the heart.

PDK1 is required for activation of the cardiac mTOR/p70S6K pathway by leucine independently of PKB/Akt. Leucine activates p70S6K in several tissues (15, 18, 29, 30, 39). Here, we confirmed this effect of leucine for the first time in the perfused adult heart. Leucine action was also verified in primary cultured adult and neonatal cardiomyocytes (see supplemental data), demonstrating that this effect was independent of the cardiac development phase. Valine and isoleucine, the two other branched-chain amino acids, did not mimic leucine action on p70S6K (see supplemental data). By contrast with the effect of insulin, p70S6K activation by leucine did not involve PKB/Akt activation. This has already been reported in many other tissues (2, 13, 19, 21, 30, 43). The main goal of the present study was the evaluation of the role of PDK1 in leucine effect. In the absence of PDK1, leucine, similarly to insulin, was no more able to induce p70S6K activation. This was accompanied by the absence of phosphorylation of mTOR on Ser2448 and p70S6K on Thr389, the mTOR phosphorylation site. These results were expected for the insulin treatment, this hormone acting through PKB/Akt, but not for leucine action. Our study clearly showed that PDK1 is required for the leucine-mediated phosphorylation of mTOR on Ser2448 and, subsequently, of p70S6K on Thr389, this leucine effect being totally independent of PKB/Akt (Fig. 10).

Activation of the mTOR/p70S6K pathway by leucine correlates with PRAS40 phosphorylation, which required PDK1. Knowing that amino acids have been proposed to activate a...
PI3K of class 3 (20, 21), we could suggest that PDK1 is recruited and/or activated by leucine in a PI3K-dependent manner and therefore is involved in the recruitment and activation of mTOR/p70S6K (Fig. 10). Several proteins, including the G proteins Rheb and Rag and PRAS40, could act as intermediate partners through which PDK1 activates mTOR under leucine treatment (2, 49–51). In the present work, we showed that leucine, in the absence of PKB/Akt activation, induced PRAS40 phosphorylation on Thr246, the site phosphorylated by PKB/Akt and responsible for mTOR activation under insulin treatment. Moreover, this leucine-mediated PRAS40 phosphorylation is entirely dependent on the presence of PDK1. 14-3-3 being involved downstream PRAS40 phosphorylation, it was important to verify that the absence of leucine effect on mTOR/p70S6K in PDK1−/−/− mice was not due to modification of 14-3-3 protein level. We observed by immunoblots that 14-3-3 expression was not altered in PDK1−/−/− hearts (data not shown). Considering all our results, we postulate that leucine induced the recruitment and activation of mTOR/p70S6K via the phosphorylation of PRAS40 in a PDK1-dependent manner. Furthermore, this leucine-mediated PRAS40 phosphorylation is entirely dependent on the presence of PDK1. 14-3-3 phosphorylation in PDK1−/−/− mice was not due to modification of 14-3-3 protein level. We observed by immunoblots that 14-3-3 expression was not altered in PDK1−/−/− hearts (data not shown). Considering all our results, we postulate that leucine induced the recruitment and activation of mTOR/p70S6K via the phosphorylation of PRAS40 in a PDK1-dependent manner. However, the implication of other partners than PRAS40, like Rag or Rheb, cannot be excluded.

Different mechanisms of PRAS40/mTOR/p70S6K activation by PDK1 under leucine and insulin treatment. Using the PDK1 L155E KI mice, we were able to further demonstrate that leucine stimulated the PRAS40/mTOR/p70S6K-signaling pathway in a different manner than insulin. Indeed, L155E mutation did not preclude the insulin-induced PKB/Akt activation and PRAS40/mTOR/p70S6K phosphorylation. In contrast, the PDK1 L155E mutation prevented leucine from inducing similar stimulation. This demonstrates the importance of the PIF-binding pocket of PDK1 in leucine signaling, whereas the pleckstrin homology domain of PDK1 is involved only in the proximal insulin signaling, namely PKB/Akt. This is in agreement with recent findings showing that a mutated form of the pleckstrin homology domain of PDK1 was unable to activate PKB/Akt downstream of insulin, whereas it still induced p70S6K activation under nutrient stimulation (4). The PIF-binding pocket of PDK1 is known to be involved in the regulation of several protein kinases of the AGC kinase family, except PKB/Akt but including p70S6K itself. Using rapamycin, we excluded the putative implication of p70S6K in the leucine-induced phosphorylation of PRAS40. Moreover, use of specific PKA and PKC inhibitors (see supplemental data) ruled out any implication of these two protein kinases in the leucine-mediated p70S6K activation. So, with PKB/Akt, p70S6K, PKA, and PKC.
PKC disqualified, the candidate playing the role of PRAS40 kinase under leucine treatment should be found in the remaining PKD1 substrates from the AGC kinase family (Fig. 10). Another member of this family, the serum- and glucocorticoid-regulated kinase (SGK3) was also potentially interesting because it has been related to amino acid transport (35). However, insulin, but not leucine, increased SGK3 phosphorylation in perfused hearts (data not shown).

Mechanisms of phosphorylation of mTOR. The use of the two genetic models of PKD1 also provided partial clarification on the mechanisms involved in mTOR phosphorylation by insulin. Recently, two studies showed that p70S6K by itself could be the kinase responsible for the phosphorylation of Ser2448, setting p70S6K upstream of mTOR in a potential feedback loop (11, 24). Our results using PKD1 L155E KI mouse hearts are in opposition to this hypothesis. In these hearts, insulin was still able to induce phosphorylation of mTOR on Ser2448 without any significant activation of p70S6K, its ultimate phosphorylation on Thr389 being prevented by the PKD1 mutation L155E (9, 12). This rules out any participation of p70S6K in mTOR phosphorylation and questions the identity of the mTOR kinase downstream of PKB/Akt in the insulin pathway. Whatever the identity of this PKD1/PKB/Akt-dependent mTOR kinase under insulin treatment, the protein kinase responsible for the phosphorylation of mTOR on Ser2448 when stimulated by leucine is probably different and/or differentially activated.

Leucine and glutamine have opposite effects on p70S6K in the adult heart. Glutamine decreased both basal and insulin-induced p70S6K activation and Thr389 phosphorylation in perfused adult hearts (Fig. 10). This correlated with decreased mTOR phosphorylation. One hypothesis to explain this inhibitory effect of glutamine could be the decrease in intracellular leucine content, the transport of these two amino acids being jointly and inversely regulated. We measured the leucine content of perfused hearts and did not see any significant effect of glutamine on this content (untreated hearts: 12.5 ± 4.2; insulin-treated hearts: 8.1 ± 4.3; leucine/g tissue, n = 3, not significant). Whatever the mechanism involved, we confirmed the inhibitory effect of glutamine in primary cultured adult cardiomyocytes (see supplemental data). The inhibitory effect of glutamine is at variance with its stimulatory action in neonatal cardiomyocytes (53). We confirmed that glutamine stimulates p70S6K phosphorylation in neonatal rat cardiomyocytes (see supplemental data). The different responses of adult and neonatal cardiomyocytes to glutamine are puzzling, and several questions remain unanswered. How does glutamine activate mTOR/p70S6K during cardiac development but inhibit it when the heart reaches its adult phase? What is the physiological importance of these divergent effects of glutamine? Clearly, further studies are required to elucidate the role played by glutamine in nutrient transduction signaling.

The main conclusions of this study are the following. 1) PKD1 is required for the stimulation of the mTOR/p70S6K pathway by leucine in the heart. 2) These leucine effects correlate with the phosphorylation of PRAS40, a crucial mTOR regulator. 3) The molecular mechanisms involving PKD1 in the stimulation of the PRAS40/mTOR/p70S6K pathway downstream of insulin and leucine are different. This study provides clear genetic evidence that PKD1 plays multiple roles in the regulation of the mTOR/p70S6K pathway known to be involved in the control of cell size and growth. This multiple implication could partially explain the severity of the cardiac phenotype of PKD1−/− compared with that usually found in cardiac-specific knockout of the other proximal constituents of the insulin-signaling pathway. Knowing that we cannot exclude a role of PKD1 in some other regulatory functions of leucine and nutrients, this makes PKD1 a putative key target to treat various cardiomyopathies.

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