

Insulin-Mediated Phosphorylation of the Proline-Rich Akt Substrate PRAS40 Is Impaired in Insulin Target Tissues of High-Fat Diet–Fed Rats

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Clinical insulin resistance is associated with decreased activation of phosphatidylinositol 3'-kinase (PI3K) and its downstream substrate protein kinase B (PKB)/Akt. However, its physiological protein substrates remain poorly characterized. In the present study, the effect of in vivo insulin action on phosphorylation of the PKB/Akt substrate 40 (PRAS40) was examined. In rat and mice, insulin stimulated PRAS40-Thr246 phosphorylation in skeletal and cardiac muscle, the liver, and adipose tissue in vivo. Physiological hyperinsulinemia increased PRAS40-Thr246 phosphorylation in human skeletal muscle biopsies. In cultured cell lines, insulin-mediated PRAS40 phosphorylation was prevented by the PI3K inhibitors wortmannin and LY294002. Immunohistochemical and immunofluorescence studies showed that phosphorylated PRAS40 is predominantly localized to the nucleus. Finally, in rats fed a high-fat diet (HFD), phosphorylation of PRAS40 was markedly reduced compared with low-fat diet–fed animals in all tissues examined. In conclusion, the current study identifies PRAS40 as a physiological target of in vivo insulin action. Phosphorylation of PRAS40 is increased by insulin in human, rat, and mouse insulin target tissues. In rats, this response is reduced under conditions of HFD-induced insulin resistance. *Diabetes* 55:3221–3228, 2006

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DAPI, 4',6-diamidino-2-phenylindole; ERK, extracellular signal-related kinase; GSK, glycogen synthase kinase; HFD, high-fat diet; LFD, low-fat diet; mTOR, mammalian target of rapamycin; PI3K, phosphatidylinositol 3'-kinase; PKB, protein kinase B; PRAS40, proline-rich Akt substrate 40; TBS, Tris-buffered saline.

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Insulin resistance and type 2 diabetes are associated with impaired insulin action in peripheral tissues like skeletal muscle, adipose tissue, liver, and the heart (1). Insulin action is initiated by binding of insulin to its receptor, leading to activation and phosphorylation of the receptor tyrosine kinase, which in turn phosphorylates several endogenous substrates, including the insulin receptor substrate proteins (1). Tyrosine phosphorylation of the insulin receptor substrate proteins facilitates the binding and activation of phosphatidylinositol 3'-kinase (PI3K), thus catalyzing the formation of phosphatidylinositol 3,4,5-trisphosphate and providing a platform for the binding and activation of protein kinase B (PKB)/Akt (1). Numerous studies have linked PKB/Akt to the regulation of glucose metabolism, cell growth, and antiapoptosis (2,3). However, the endogenous substrates regulating these responses are only starting to become characterized (4).

The PKB/Akt protein kinase phosphorylates proteins on serine (S/Ser) or threonine (T/Thr) residues within a RxxRxxpS/pT motif (5). The use of phospho-specific antibodies recognizing this PKB/Akt consensus sequence led to the identification of multiple novel proteins, including proline-rich Akt substrate 40 (PRAS40; also known as Akt1 substrate 1 [Akt1S1]) (6,7). PRAS40 is ubiquitously expressed and appears to be localized to the nucleus (7,8). In response to growth factors, PRAS40 is phosphorylated on Thr246 via PI3K- and PKB/Akt-dependent signaling pathways (6,8). In vitro, phosphorylation of PRAS40 facilitates the binding of 14-3-3 proteins, and this protein complex has been implicated in nerve growth factor-mediated neuroprotection from ischemia (8). Although PRAS40 is phosphorylated in response to stimulation of cultured cells with insulin in vitro (6,9), it is as yet unknown whether this protein is involved in physiological insulin action. In the present study, we analyzed whether PRAS40-Thr246 phosphorylation is induced in response to in vivo insulin treatment in various insulin target tissues in humans, rats, and mice. Furthermore, we studied whether this response is altered under conditions of high-fat diet (HFD)-induced insulin resistance.

RESEARCH DESIGN AND METHODS

Materials and cell lines. A14 cells are NIH3T3 fibroblasts overexpressing the human insulin receptor (10). H9c2-E2 cells are cardiomyocytes overexpressing the human insulin receptor (11). Wortmannin, LY294002, and rapamycin

were purchased from Calbiochem, Darmstadt, Germany. U0126 was obtained from Promega Benelux, Leiden, the Netherlands. Anti-phospho (Ser/Thr)-Akt-substrate (no. 9611), anti-phospho-Ser473-PKB/Akt (nos. 9271 and 4051), anti-phospho-Thr421/Ser424-p70 S6 kinase (no. 9204), anti-phospho-Thr202/Tyr204-extracellular signal-related kinase (ERK) 1/2 (no. 9101), and anti-phospho-glycogen synthase kinase (GSK) 3 α/β -Ser21/Ser9 (no. 9931) were from Cell Signaling Technology (Beverly, MA); anti-phospho-PRAS40-Thr246 (no. 44-100G) and anti-phospho-AS160-Thr642 (44-1071G) were from Bioscience International (Camarillo, CA); and anti-AS160 (ab5909) was from Abcam plc (Cambridge, U.K.). PKB/Akt antiserum was kindly provided by Dr. B. Burgering (Utrecht University, Utrecht, the Netherlands). PRAS40 antibody was kindly provided by Dr. R. Roth (Stanford University, Stanford, CA) (6).

Human biopsies. Muscle biopsies were obtained from 10 obese subjects with type 2 diabetes (8 female and 2 male subjects, aged 54 ± 3 years, with fasting plasma glucose 11.1 ± 0.8 mmol/l, HbA_{1c} $7.7 \pm 0.4\%$, and BMI 40.2 ± 1.6 kg/m²) participating in a clinical study that was approved by the medical ethical committee of the Leiden University Medical Center. Patients used at least 30 units of exogenous insulin; eight patients also used metformin, and two patients used rosiglitazone. Written informed consent was obtained from the patients following explanation of the experimental procedures. Biopsies were collected following an overnight fast (baseline) and 30 min after initiation of a hyperinsulinemic-euglycemic clamp (insulin infusion rate: 40 mU/m² per min) (12,13). Muscle biopsies were taken from the vastus lateralis muscle after localized anesthesia with 1% lidocaine with a modified Bergström needle and snap frozen in dry ice-chilled isopentane (14).

Animals. The investigation conformed to the *Guide for the Care and Use of Laboratory Animals*, as published by the National Institutes of Health (NIH publ. no. 85-23, revised 1996) and the regulations of the institutional animal care and use committee. Adult male Wistar WU rats ($n = 20$; mean body weight 302 ± 6 g) were obtained from Harlan CBP (Horst, the Netherlands). Animals were fed an HFD or a low-fat diet (LFD) for 7 weeks as described (15). Rats were fasted for 6 h, and insulin stimulation was induced through intraperitoneal injection of 10 units/kg body wt insulin (Actrapid 100 units/ml; Novo Nordisk, Alphen Aan Den Rijn, the Netherlands) 30 min before being killed by decapitation. Animals were considered insulin stimulated when the plasma insulin levels exceeded 2 nmol/l. Similar insulin levels were achieved in HFD- and LFD-fed rats (data not shown). Control animals received an intraperitoneal injection with saline. Trunk blood was collected for glucose determinations using a HemoCue glucose analyzer (Angelholm, Sweden). Plasma insulin levels were determined as described (15). Male C57BL6/J mice ($n = 12$; mean body weight 23.2 ± 2 g), obtained from Charles River (Maastricht, the Netherlands), were fasted overnight and anesthetized by intraperitoneal injection with a combination of 6.25 mg/kg acetylpromazine (Sanofi Santé Nutrition Animale, Libourne Cedex, France), 6.25 mg/kg midazolam (Roche, Mijdrecht, the Netherlands), and 0.3125 mg/kg fentanyl (Janssen-Cilag, Tilburg, the Netherlands). Insulin was infused at 24.5 pmol \cdot kg body wt⁻¹ \cdot min⁻¹ as described (16). Glucose was coinfused with insulin at 105 μ mol/min to maintain euglycemia (4.7 ± 0.7 mmol/l). Blood glucose was measured with the FreeStyle hand glucose measurer (Therasense; Disetronic Medical Systems, Vianen, the Netherlands). At 10, 15, and 20 min following initiation of the insulin infusion, mice were killed by cervical dislocation. Control animals were killed following a 20-min infusion with PBS. Organs were removed and either immersion fixed in 4% buffered formaldehyde solution or snap frozen in dry ice-chilled isopentane and stored at -80°C until further use.

Western blotting. Homogenates of tissue biopsies were prepared in 30 mmol/l Tris.Cl, pH 7.5, 150 mmol/l NaCl, 0.5% Triton X100, 0.5% sodium deoxycholate, 1% SDS, 1 mmol/l Na₃VO₄, 10 mmol/l NaF, and protease inhibitors (Complete; Roche). Cultured cells were lysed in 100 mmol/l Tris.Cl, pH 6.8, 3% SDS, and 10% glycerol. Homogenates were cleared by centrifugation (13,200 rpm; 15 min, 4°C). Protein content was determined using a bicinchoninic acid protein assay kit (Pierce, Rockford, IL). Expression and phosphorylation of proteins was determined by SDS-PAGE and immunoblotting (15). Bound antibodies were visualized by enhanced chemiluminescence and quantified by densitometry analysis of scanned films (ImageJ 1.34S; National Institutes of Health, Bethesda, MD).

Immunohistochemistry. Fixed tissues were prepared by routine paraffin embedding (Histowax). Sections (6 μ m) were cut and mounted on 3-aminopropyltriethoxysilane-coated slides (Menzel, Darmstadt, Germany) to prevent detachment due to the high temperature during antigen retrieval treatment, which was necessary for the visualization of phospho-PRAS40-Thr246 antibody. After deparaffinization and rehydration, the slides were heated (12 min at 900 W) in 0.01 mol/l citrate buffer (pH 6.0). The sections were allowed to cool down at room temperature, were rinsed extensively with Tris-buffered saline (TBS; 50 mmol/l Tris.Cl and 500 mmol/l NaCl, pH 7.6), and were incubated overnight with the phospho-PRAS40-Thr246 antibody diluted (1:1,000) in TBS containing 0.5% Triton X100, pH 7.6, at 4°C . After several

washes in TBS, sections were incubated with biotinylated anti-rabbit IgG (1.5 h at room temperature; Vector Laboratories, Burlingame, CA) and diluted (1:250) in TBS, followed by avidin-biotin-peroxidase (1 h at room temperature, 1:400, ABC Elite kit; Vector Laboratories). Tissue-bound peroxidase was visualized with the 3,3'-diaminobenzidine tetrahydrochloride chromogen reaction (7.5 mg diaminobenzidine tetrahydrochloride, 5 μ l 30% H₂O₂ in 15 ml 50 mmol/l Tris.Cl, pH 7.6) for 10 min at room temperature. Sections were then rinsed with distilled water, dehydrated in a graded series of ethanol, cleared in xylene, and coverslipped with Entellan (Merck, Darmstadt, Germany).

Immunofluorescence. Cells grown on coverslips (18 mm) were rinsed with ice-cold PBS and fixed in 3.7% formaldehyde in TBS (25 mmol/l Tris.Cl, 100 mmol/l NaCl, 5 mmol/l KCl, 0.7 mmol/l CaCl₂, and 0.5 mmol/l MgCl₂, pH 7.4) (15 min at room temperature). To quench residual cross-linking, coverslips were rinsed with 50 mmol/l NH₄Cl in TBS (10 min at room temperature) and subsequently permeabilized with 0.1% Triton X-100 in TBS (5 min at room temperature). Cells were washed with 0.2% BSA/TBS, blocked with 0.2% BSA/TBS (30 min at room temperature), and incubated with primary antibodies diluted (1:100) in 0.2% BSA/TBS (overnight, 4°C). After 0.2% BSA/TBS washes, coverslips were incubated with appropriate secondary antibodies diluted (1:100) in 0.2% BSA/TBS (2 h at room temperature). Subsequently, coverslips were cleaned with 0.2% BSA/TBS and TBS and milliQ and mounted in 4',6-diamidino-2-phenylindole [DAPI]-containing Vectashield solution (Vector Laboratories). Fluorescence was detected using a Leica DM-RXA microscope (filters DAPI, fluorescein isothiocyanate, and TRITC [tetramethylrhodamine isothiocyanate]).

Data analysis. Data are expressed as means \pm SE. Differences between groups were determined by unpaired Student's two-tailed *t* test, using Macintosh version 11.0.4 (SPSS, Chicago, IL). $P < 0.05$ was considered statistically significant.

RESULTS

Characterization of endogenous PKB/Akt substrates in insulin target tissues. The effects of in vivo insulin stimulation on the phosphorylation of endogenous PKB/Akt substrates in rat liver, adipose tissue, and cardiac and skeletal muscle were assessed by immunoblotting with the phospho (Ser/Thr)-PKB/Akt-substrate antibody. In all examined tissues, insulin induced the phosphorylation of a ~ 30 -kDa (p30) and a ~ 40 -kDa (p40) protein (Fig. 1A). Only in cardiac and skeletal muscle was phosphorylation of a ~ 160 -kDa (p160) protein observed. Reprobing the stripped membranes with appropriate antibodies identified p30 as phosphorylated ribosomal protein S6. This was subsequently confirmed by cell fractionation and isolating the ribosomal 40S and 60S subunits by sucrose gradient fractionation (data not shown). The p160 protein was identified as Akt substrate 160 (AS160) (data not shown). p40 migrated at a molecular weight similar to GSK3 β and PRAS40 (data not shown). Isoelectric focusing of liver homogenates followed by SDS-PAGE and immunoblotting showed that a 40-kDa PKB/Akt substrate immunoreactivity was predominantly found at the acidic region at an isoelectric point corresponding to PRAS40 (calculated isoelectric point 4.7) rather than GSK3 β (calculated isoelectric point 9.0) (Fig. 1B). Reprobing the stripped filter with the phospho-PRAS40-Thr246 antibody confirmed this protein as phosphorylated PRAS40 (Fig. 1B).

Insulin induces PRAS40-Thr246 phosphorylation.

In vivo. Whereas ribosomal protein S6 and AS160 have been implicated in the regulation of protein synthesis and glucose transport, respectively, little is known on the function of PRAS40 in insulin action. Therefore, we decided to focus on regulation of this protein. Figure 2A shows that PRAS40-Thr246 phosphorylation was induced in mouse liver and skeletal and cardiac muscle within 10 min after initiation of an insulin infusion (24.5 pmol \cdot kg body wt⁻¹ \cdot min⁻¹). The onset of PRAS40-Thr246 phosphorylation in these murine tissues correlated with the induction of PKB/Akt-Ser473 phosphorylation, whereas

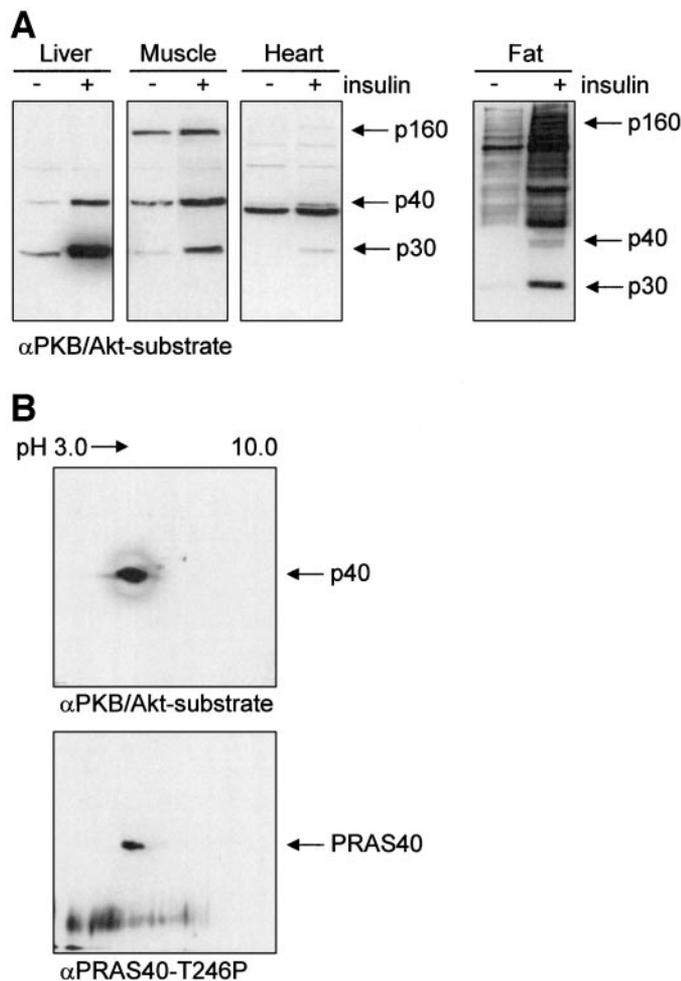


FIG. 1. A: Phosphoproteins detected by the PKB/Akt substrate antibody in rat liver, skeletal muscle, cardiac ventricular tissue, and adipose tissue following intraperitoneal saline (-) or insulin (+) injection. B: Identification of p40 as PRAS40. Immunoblots prepared after isoelectric focusing of liver homogenate from insulin-treated rats were incubated with the PKB/Akt substrate antibody and the PRAS40 antibody, respectively.

protein expression levels of PRAS40 and PKB/Akt were similar at the various time points examined (Fig. 2A). In rats, intraperitoneal injection of insulin (30 min, 10 units/kg body wt) stimulated PRAS40-Thr246 phosphorylation in the liver, heart, skeletal muscle, and adipose tissue (Fig. 2B). Protein expression of PRAS40 was similar between saline and insulin-injected samples. Also, in skeletal muscle biopsies obtained from obese subjects with type 2 diabetes, a 2.5 ± 0.4 -fold increase ($n = 8$; $P < 0.02$) in PRAS40-Thr246 phosphorylation was found 30 min after initiation of the hyperinsulinemic-euglycemic clamp at an insulin infusion rate of 40 mU/m² per min (Fig. 2C). The increase in PRAS40-Thr246 phosphorylation in response to hyperinsulinemia was paralleled by a 1.9 ± 0.3 -fold increase in AS160-Thr642 phosphorylation ($n = 8$; $P < 0.01$) and could not be ascribed to differences in PRAS40 and AS160 protein expression, respectively (Fig. 2C).

In vitro. Studies in cultured rat H9c2-E2 cardiomyocytes showed a 2.6 ± 0.1 -fold induction of PRAS40-Thr246 phosphorylation within 1 min and maximal stimulation (3.9 ± 0.3 -fold over basal) within 2 min after the addition of insulin (Fig. 3). Also, in A14 fibroblasts and 3T3L1 adipocytes (data not shown), induction of PRAS40-Thr246

phosphorylation was observed within 1 min after the addition of insulin. The presence of the PI3K inhibitors wortmannin or LY294002 (data not shown) abrogated the phosphorylation of PRAS40 and PKB/Akt in response to insulin stimulation (Fig. 3A). Inhibition of the mammalian target of rapamycin (mTOR)/p70 S6 kinase and mitogen-activated protein kinase kinase (MEK)/ERK1/2 pathways by rapamycin and U0126, respectively, had no effect on phosphorylation of PKB/Akt or PRAS40 after insulin stimulation (Fig. 3B), indicating that PRAS40 is a distal component of the class I PI3K/PKB/Akt-mediated signaling pathway rather than the class III PI3K/mTOR pathway.

Phosphorylated PRAS40 is localized in the nucleus. Immunohistochemical staining of liver and cardiac ventricular tissue from insulin-injected rats with the phospho-PRAS40-Thr246 antibody shows a nuclear localization of the protein both in hepatocytes and cardiomyocytes (Fig. 4). Immunofluorescence labeling of A14 and H9c2-E2 cells with phospho-PRAS40-Thr246 antibodies substantiated the predominant nuclear localization of PRAS40. In response to insulin stimulation, the nuclear phospho-PRAS40-Thr246 immunoreactivity was markedly increased. Some PRAS40 staining was observed in the cytoplasm in the vicinity of phosphorylated PKB/Akt (Fig. 5B). The insulin-mediated increase in phospho-PRAS40-Thr246 immunoreactivity was prevented in the presence of LY294002 (Fig. 5).

Impaired insulin-mediated phosphorylation in tissues from HFD-fed rats. We next examined whether the induction of PRAS40-Thr246 phosphorylation by insulin was altered under conditions of HFD-induced insulin resistance in rats. Tissues were collected from the same rats already described in a previous study, where we showed that HFD induced impaired glucose tolerance, myocardial insulin resistance, and skeletal muscle and hepatic steatosis (15). Following a 7-week exposure to a HFD, fasting blood glucose values were increased compared with rats fed an isocaloric LFD ($P < 0.005$; Table 1). No changes in body weight and fasting plasma insulin levels were observed between LFD- and HFD-fed rats (Table 1).

In skeletal muscle, insulin versus saline stimulated PRAS40-Thr246 phosphorylation by 19.6-fold in LFD-fed rats and 9.0-fold in HFD-fed rats ($P < 0.05$), respectively (Fig. 6A; Table 2). Also, PKB/Akt Ser473 phosphorylation and AS160-Thr642 phosphorylation were increased in skeletal muscle from insulin-injected LFD-fed rats but not in muscles from HFD-fed animals (Fig. 6A). Protein expression of PRAS40 and PKB/Akt did not differ among the experimental groups (Fig. 6A). In the heart, insulin stimulated PRAS40-Thr246 phosphorylation by 6.7-fold in LFD-fed rats and 2.2-fold in HFD-fed rats ($P < 0.05$) (Fig. 6B; Table 2). In the liver, a 2.5-fold increase in PRAS40-Thr246 phosphorylation was observed in LFD-fed animals, and this response was completely abrogated in livers from HFD-fed rats ($P < 0.05$) (Fig. 6C; Table 2). In adipose tissue, insulin stimulated PRAS40-Thr246 phosphorylation by 4-fold in LFD-fed rats and by 1.5-fold in HFD-fed rats ($P < 0.05$) (Fig. 6D; Table 2). Rats exposed to the same HFD for a period of 10–20 weeks also showed insulin resistance for the induction of PRAS40-Thr246 phosphorylation (data not shown).

In all tissues, the insulin-mediated changes in PRAS40-Thr246 phosphorylation and the blunted response in tissues from HFD-fed rats were paralleled by comparable changes in PKB/Akt Ser473 phosphorylation. Only in skel-

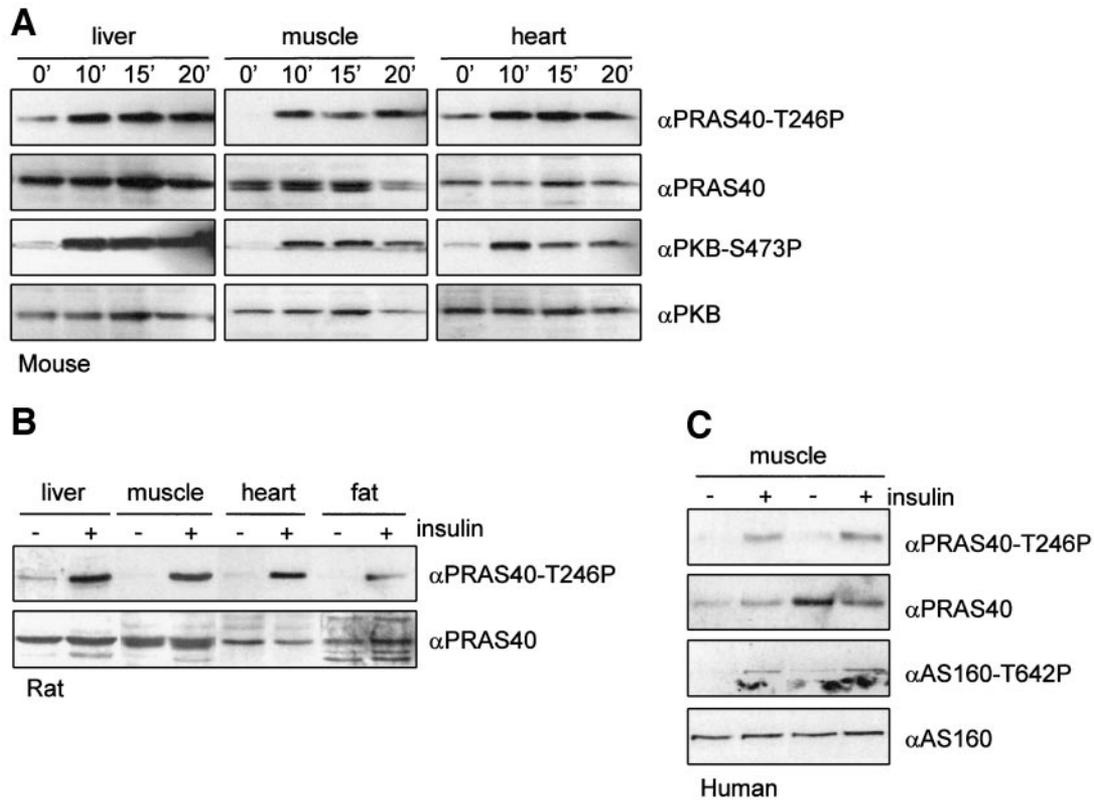


FIG. 2. A: Phosphorylation and protein expression of PRAS40 and PKB/Akt in mouse liver, skeletal muscle, and heart following in vivo insulin infusion during 10, 15, and 20 min. Control animals received PBS (0). **B:** Phosphorylation and protein expression of PRAS40 in rat liver, skeletal and cardiac muscle, and adipose tissue following intraperitoneal saline (-) or insulin (+) injection. **C:** Phosphorylation and protein expression of PRAS40 and AS160 in human skeletal muscle biopsies under basal and hyperinsulinemic-euglycemic conditions. All experiments are representative of at least three independent observations.

etal muscle could we reproducibly detect insulin-induced AS160-Thr642 phosphorylation. Therefore, we analyzed the other tissues for insulin-mediated phosphorylation of another PKB/Akt substrate (i.e., GSK3). In heart and liver, the induction of GSK3 α/β Ser21/9 phosphorylation in

response to insulin closely resembled the induction of PKB/Akt and PRAS40 phosphorylation (Fig. 6B and C). In adipose tissue, basal phosphorylation of GSK3 α/β was elevated in adipose tissue from HFD-fed rats, and while insulin stimulated GSK3 α/β Ser21/9 phosphorylation in

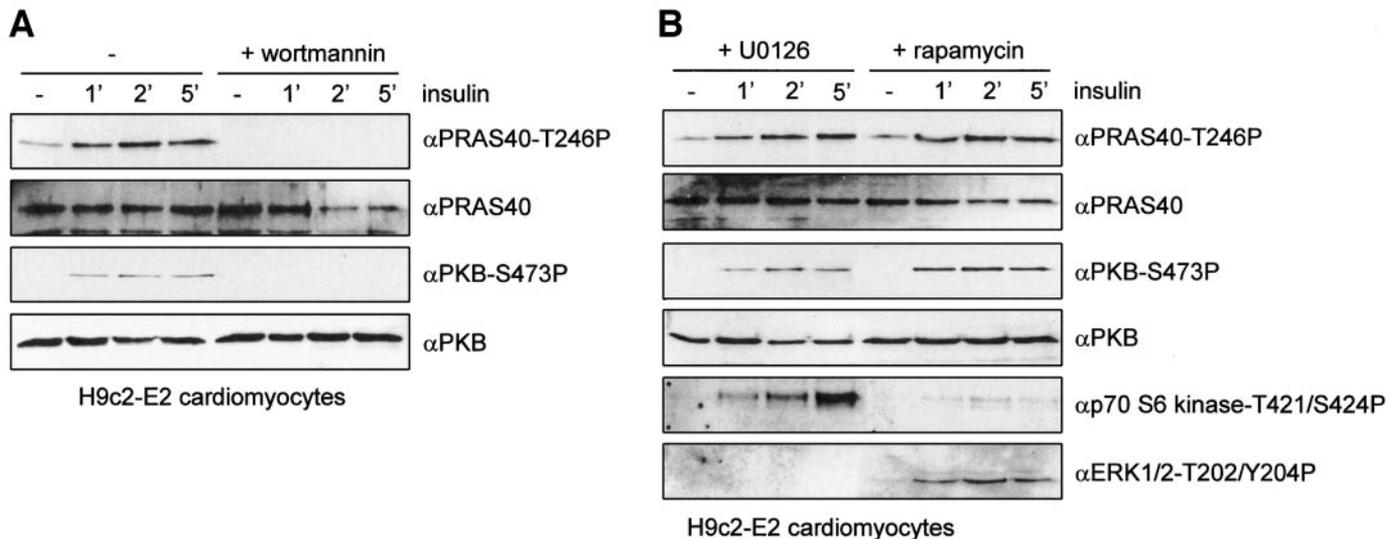


FIG. 3. Effect of wortmannin (A) and U0126 and rapamycin (B) on the phosphorylation of PRAS40-Thr246 following stimulation of H9c2-E2 cardiomyocytes with 100 nmol/l insulin. Comparable exposures for PRAS40-Thr246 blots are shown. When indicated, cells were pretreated with wortmannin (100 nmol/l, 30 min), U0126 (10 μ mol/l, 15 min), or rapamycin (100 nmol/l, 15 min). Effectiveness of wortmannin, U0126, and rapamycin was verified by reprobings the membranes with phospho-specific antibodies for PKB/Akt, ERK1/2, and p70 S6 kinase, respectively. Equal loading was verified by reprobings the blots with PKB/Akt antibody.

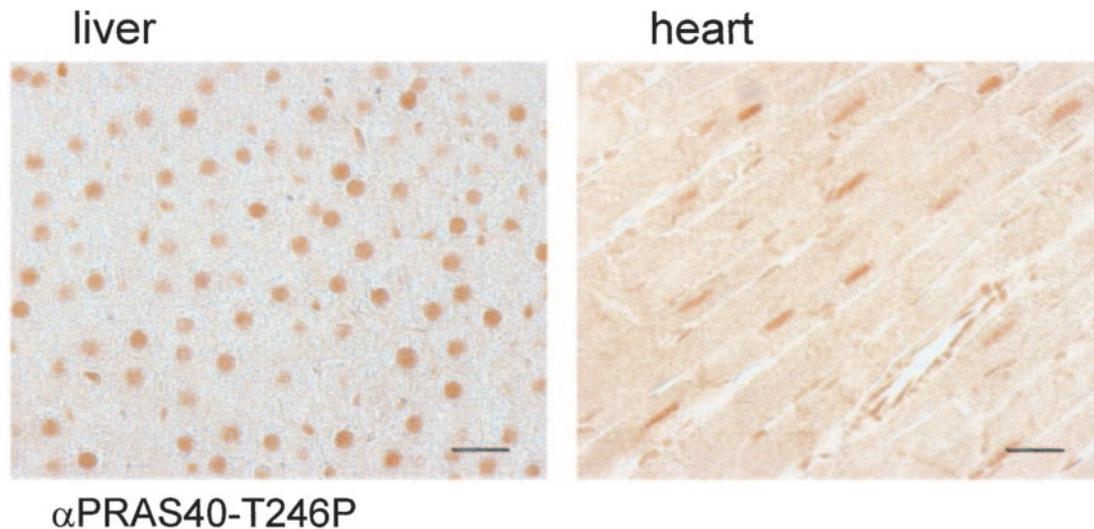


FIG. 4. Immunohistochemical staining of rat liver and cardiac ventricular tissue sections with PRAS40-Thr246 antibody. Rats received an intraperitoneal injection with insulin 30 min before they were killed. Photographs are representative of four independent experiments. The scale bar indicates 25 μ m.

LFD-fed adipose tissue, this stimulatory effect was absent in adipose tissue from HFD rats (Fig. 6D). Collectively, these observations agree with a situation in which the phosphorylation state of PRAS40 is predominantly regulated through insulin-mediated activation of PKB/Akt.

DISCUSSION

The serine/threonine kinase PKB/Akt has been identified as a crucial mediator of insulin action. Activation of this kinase contributes, among others, to the regulation of glucose uptake, glycogen metabolism, gene expression, cell survival, and proliferation and protection from apoptosis (3,17). The use of phospho-specific antibodies recognizing the PKB/Akt consensus phosphorylation site $RxRxx(pS/pT)$ has led to the identification of several novel putative substrates for PKB/Akt (4), including AS160 (18), ATP-citrate lyase (19), PIKfyve (20), Wnk1 (21), and PRAS40 (6,7), in addition to already known substrates like the forkhead/FOXO transcription factors, endothelial nitric oxide synthase, mTOR, GSK3, and Bad (2). However, when analyzing target tissues for insulin action, among the most prominent proteins that are recognized by the PKB/Akt-substrate antibody was p40, which was identified as PRAS40. In the present study, we demonstrate that phosphorylation of PRAS40 is induced in response to *in vivo* insulin stimulation in various target tissues and that phosphorylation of this protein is markedly blunted under conditions of HFD-induced insulin resistance.

PRAS40 is ubiquitously expressed in all tissues examined thus far but was originally characterized in rat H4IIE hepatoma cells, 3T3L1 adipocytes, and HeLa cell nuclear extracts using the PKB/Akt substrate antibody (6,7,9). Interestingly, phosphorylation of an as yet unidentified ~46-kDa protein recognized by the PKB/Akt substrate antibody has been reported in insulin-stimulated human skeletal muscle, and phosphorylation of this protein is impaired in patients with type 2 diabetes. Given the induction of PRAS40-Thr246 phosphorylation by insulin in human skeletal muscle observed here, it seems plausible that the 46-kDa protein is similar to PRAS40 (22). In the present study, a matched healthy control group was not available. However, improvement of insulin sensitivity

induced by weight loss was accompanied by increased phosphorylation of PRAS40 (from 2.5- to 3.8-fold stimulation [$P < 0.02$] in response to 30 min hyperinsulinemia) (I.M.J., unpublished data), indicating that PRAS40-Thr246 phosphorylation is a bona fide marker for insulin sensitivity in human skeletal muscle.

Phosphorylation of PRAS40 was also observed in response to other growth factors, including platelet-derived growth factor, epidermal growth factor, and nerve growth factor (6,8,23), and this process was completely abrogated in mouse embryonic fibroblasts lacking Akt1 and Akt2 (6). Inhibitors of PI3K, but not rapamycin and MEK1/2 inhibition, prevented the induction of PRAS40 phosphorylation by insulin. In various mice tissues examined (skeletal muscle, heart, and liver), the onset of PRAS40 phosphorylation following intravenous insulin infusion closely correlated with the onset of PKB/Akt phosphorylation (data not shown). Furthermore, in tissues from HFD-fed rats, the reduction in PRAS40 phosphorylation was accompanied by reduced phosphorylation of PKB/Akt and its substrates AS160 and GSK3 α/β . Collectively, these findings further identify PRAS40 as substrate for PKB/Akt also under physiological *in vivo* conditions.

Notably, we only observed reproducible insulin-mediated phosphorylation of AS160 in skeletal muscle. Analysis of various insulin target tissues with the PKB/Akt-substrate antibody suggests that p160 phosphorylation is more prominent in skeletal muscle compared with other tissues. However, pilot studies indicate that insulin is capable of increasing phosphorylation of AS160-Thr642 in isolated rat cardiomyocytes (E.B.M.N., D.M.O., unpublished data), suggesting that the failure to detect a significant increase in AS160-Thr642 phosphorylation in hearts from insulin-injected rats may be related to the sensitivity of the antibody.

Little is known about the function of the PRAS40 protein. Like others (7,8), we found that phosphorylated PRAS40 predominantly localizes to the nucleus, although in response to insulin stimulation phosphorylated PRAS40 also was found to localize in the vicinity of phosphorylated PKB/Akt in the cytoplasm. Furthermore, phosphorylated PRAS40 has been found to interact with 14-3-3 proteins

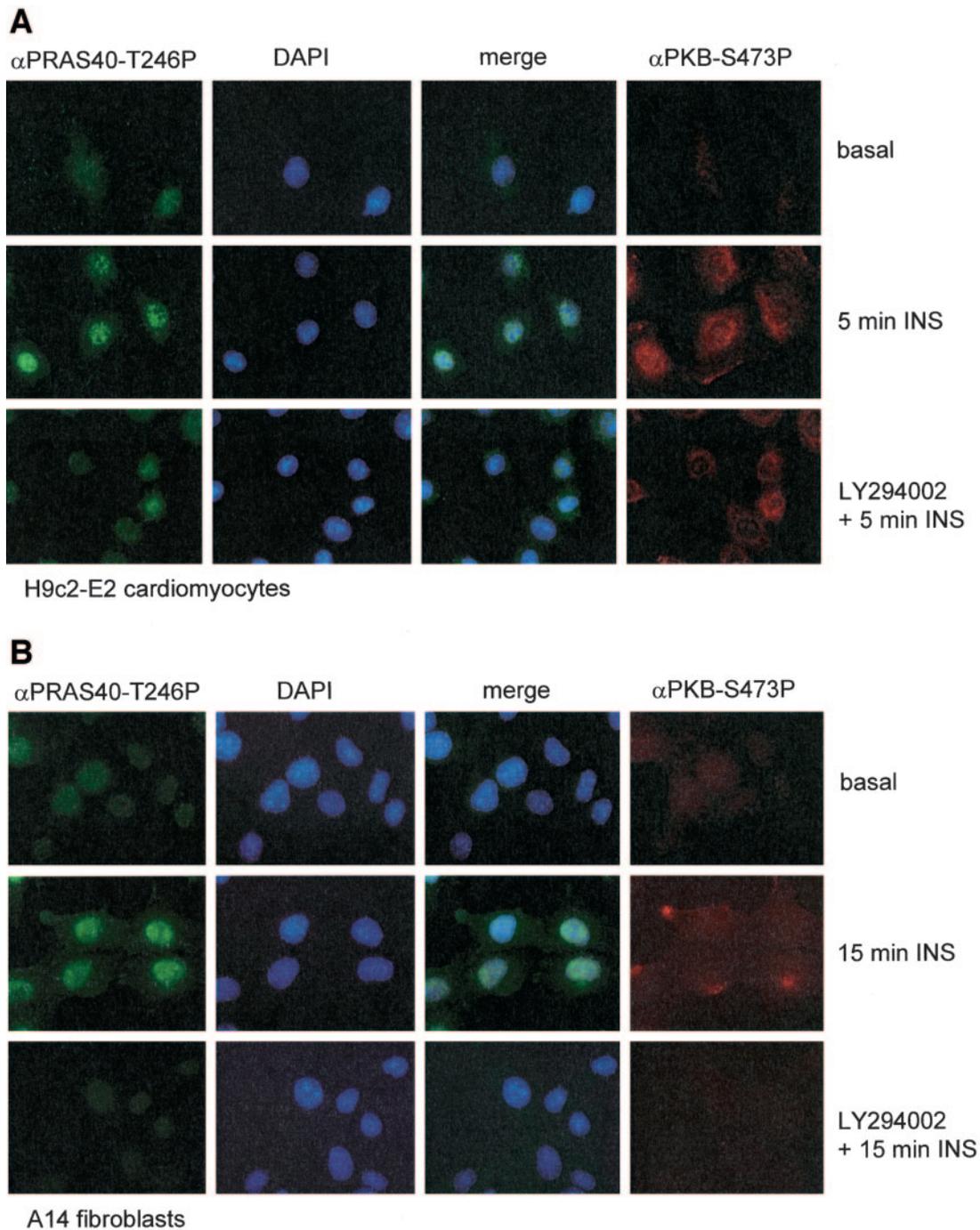


FIG. 5. Immunofluorescence staining of H9c2-E2 cardiomyocytes (A) and A14 fibroblasts (B). Cells were stimulated with 100 nmol/l insulin and where indicated pretreated with LY294002 (25 μ mol/l, 15 min). Cells were fixed and stained with phospho-specific PRAS40-Thr246 antibody followed by fluorescein isothiocyanate-conjugated anti-rabbit IgG (green) or phospho-specific PKB-Ser473 antibody followed by tetramethylrhodamine isothiocyanate (TRITC)-conjugated anti-mouse IgG (red). DNA was stained with DAPI (blue). Photographs are representative of three independent experiments.

(6,8,23). In line with a role of both 14-3-3 proteins and PKB/Akt in the regulation of cell survival (17,24), PRAS40 also has been implicated in this process, in particular in the protection of neurons against apoptosis following cerebral ischemia (8,25). Notably, PRAS40 phosphorylation in response to insulin was also observed in the arcuate nucleus of rats (data not shown). Whether PRAS40 also has antiapoptotic activity in skeletal and cardiac muscle, liver, and adipose tissue or whether it mediates another action of insulin remains to be demonstrated.

TABLE 1
Animal characteristics following a 7-week LFD or HFD intervention

	LFD	HFD
<i>n</i>	10	10
Body wt when killed (g)	446.4 \pm 6.6	454.7 \pm 6.7
Fasting blood glucose (mmol/l)	6.02 \pm 0.2	6.97 \pm 0.2*
Fasting plasma insulin (pmol/l)	385.4 \pm 34.3	350.5 \pm 46.5

Data are means \pm SE. **P* < 0.005 vs. LFD.

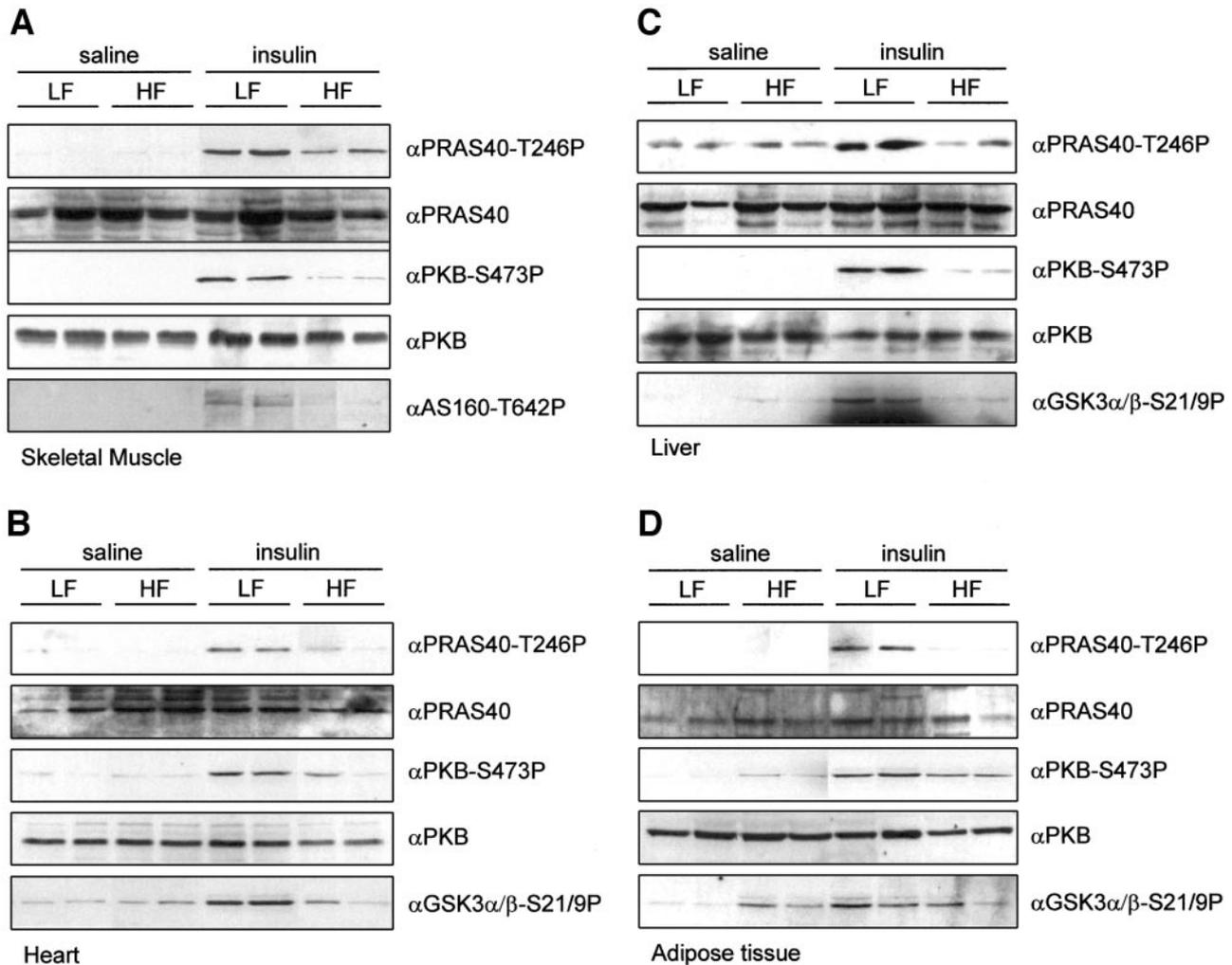


FIG. 6. Phosphorylation and protein expression of PRAS40, PKB/Akt, AS160, and GSK3 α/β in skeletal muscle (**A**), cardiac ventricular tissue (**B**), liver (**C**), and adipose tissue (**D**) from LFD- and HFD-fed rats. Thirty minutes before they were killed, rats received an intraperitoneal injection with saline or insulin (10 units/kg body wt). The immunoblots shown are representative of observations on at least four animals per experimental group.

In conclusion, the current study identifies PRAS40 as a novel physiological target of *in vivo* insulin action and a potential marker for insulin resistance. Phosphorylation of PRAS40 is increased by insulin in rat, mouse, and human insulin target tissues, and this response is reduced under conditions of HFD-induced insulin resistance. Although PRAS40 has been implicated in the protection against neuronal apoptosis after stroke, its role in insulin action is still unknown. Overexpression and knock down of PRAS40, as well as identification of interacting proteins, should provide insight into the physiological role of PRAS40.

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TABLE 2
PRAS40-Thr246 phosphorylation levels in rat tissues isolated following a 7-week LFD or HFD intervention

	LFD		HFD	
	Saline	Insulin	Saline	Insulin
<i>n</i>	4	4	4	4
Skeletal muscle	5.1 \pm 1.9	100.0 \pm 16.1*	5.7 \pm 1.9	51.1 \pm 7.6*†
Heart	15.0 \pm 6.2	100.0 \pm 17.4*	14.4 \pm 2.5	38.1 \pm 11.4†
Liver	40.7 \pm 12.3	100.0 \pm 13.8*	48.9 \pm 6.4	46.2 \pm 17.8
Adipose tissue	24.5 \pm 2.3	100.0 \pm 8.3*	38.4 \pm 8.4	57.6 \pm 12.8†

Data are means \pm SE. PRAS40-Thr246 phosphorylation was determined by densitometric analysis and is expressed as arbitrary units. **P* < 0.05 vs. saline; †*P* < 0.05 vs. LFD.

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