

# Selective Modification of Pyruvate Dehydrogenase Kinase Isoform Expression in Rat Pancreatic Islets Elicited by Starvation and Activation of Peroxisome Proliferator-Activated Receptor- $\alpha$

## Implications for Glucose-Stimulated Insulin Secretion

Mary C. Sugden, Karen Bulmer, Daniel Augustine, and Mark J. Holness

The pyruvate dehydrogenase complex (PDC) has a pivotal role in islet metabolism. The pyruvate dehydrogenase kinases (PDK1–4) regulate glucose oxidation through inhibitory phosphorylation of PDC. Starvation increases islet PDK activity (*Am J Physiol Endocrinol Metab* 270:E988–E994, 1996). In this study, using antibodies against PDK1, PDK2, and PDK4 (no sufficiently specific antibodies are as yet available for PDK3), we identified the PDK isoform profile of the pancreatic islet and delineated the effects of starvation (48 h) on protein expression of individual PDK isoforms. Rat islets were demonstrated to contain all three PDK isoforms, PDK1, PDK2, and PDK4. Using immunoblot analysis with antibodies raised against the individual recombinant PDK isoforms, we demonstrated increased islet protein expression of PDK4 in response to starvation (2.3-fold;  $P < 0.01$ ). Protein expression of PDK1 and PDK2 was suppressed in response to starvation (by 27% [ $P < 0.01$ ] and 10% [NS], respectively). We demonstrated that activation of peroxisome proliferator-activated receptor- $\alpha$  (PPAR- $\alpha$ ) by the selective agonist WY14,643 for 24 h in vivo leads to specific upregulation of islet PDK4 protein expression by 1.8-fold ( $P < 0.01$ ), in the absence of change in islet PDK1 and PDK2 protein expression but in conjunction with a 2.2-fold increase ( $P < 0.01$ ) in islet PPAR- $\alpha$  protein expression. Thus, although no changes in islet PPAR- $\alpha$  expression were observed after the starvation protocol, activation of PPAR- $\alpha$  in vivo may be a potential mechanism underlying upregulation of islet PDK4 protein expression in starvation. We evaluated the effects of antecedent changes in PDK profile and/or PPAR- $\alpha$  activation induced by starvation or PPAR- $\alpha$  activation in vivo on

glucose-stimulated insulin secretion (GSIS) in isolated islets. GSIS at 20 mmol/l glucose was modestly impaired on incubation with exogenous triglyceride (1 mmol/l triolein) (~20% inhibition;  $P < 0.05$ ) in islets from fed rats. Starvation (48 h) impaired GSIS in the absence of triolein (by 57%;  $P < 0.001$ ), but GSIS after the further addition of triolein did not differ significantly between islets from fed or starved rats. GSIS by islets prepared from WY14,643-treated fed rats did not differ significantly from that seen with islets from control fed rats, and the response to triolein addition resembled that of islets prepared from fed rather than starved rats. PPAR- $\alpha$  activation in vivo led to increased insulin secretion at low glucose concentrations. Our results are discussed in relation to the potential impact of changes in islet PDK profile on the insulin secretory response to lipid and of PPAR- $\alpha$  activation in the cause of fasting hyperinsulinemia. *Diabetes* 50:2729–2736, 2001

**T**he fate of pyruvate in islets is largely restricted to conversion to acetyl-CoA via the pyruvate dehydrogenase complex (PDC) or conversion to oxaloacetate via pyruvate carboxylase (PC), normally with approximately equal amounts of pyruvate entering each route (1–3). Flux via lactate dehydrogenase is limited by the low activity of this enzyme in islets (4). The activity of PDC, in conjunction with flux through the anaplerotic enzyme PC, links glycolysis with citrate synthesis and, in the fed state, the synthesis of malonyl-CoA. Factors that lead to PDC inactivation therefore favor acetyl-CoA production via the  $\beta$ -oxidation of long-chain fatty acids. Recent evidence suggests an important role of glucose-regulated anaplerosis and cataplerosis in triggering insulin secretion from pancreatic  $\beta$ -cells via the provision of mitochondrially derived coupling factors (5). These metabolic processes are early events in pancreatic  $\beta$ -cell activation that do not require a rise in  $Ca^{2+}$  (5). Malonyl-CoA, another component of acute fuel sensing by the pancreatic  $\beta$ -cell (6,7), inhibits mitochondrial long-chain fatty acid uptake at the level of carnitine palmitoyltransferase I and thereby promotes re-esterification. Intracellular lipid homeostasis, in part mediated via the malonyl-CoA nutrient-sensing mechanism, is essential for normal pancreatic  $\beta$ -cell function.

From the Department of Diabetes and Metabolic Medicine, Division of General and Developmental Medicine, St. Bartholomew's and the Royal London School of Medicine and Dentistry, Queen Mary, University of London, London, U.K.

Address correspondence and reprint requests to Professor Mary C. Sugden, Department of Diabetes and Metabolic Medicine, Medical Sciences Building, Queen Mary, University of London, Mile End Road, London E1 4NS, U.K. E-mail: m.c.sugden@qmw.ac.uk

Received for publication 14 December 2000 and accepted in revised form 11 September 2001.

ECL, enhanced chemiluminescence; ELISA, enzyme-linked immunosorbent assay; GSIS, glucose-stimulated insulin secretion; KRB, Krebs-Ringer bicarbonate buffer; NEFA, nonesterified fatty acid; PC, pyruvate carboxylase; PDC, pyruvate dehydrogenase complex; PDK, pyruvate dehydrogenase kinase; PPAR- $\alpha$ , peroxisome proliferator-activated receptor- $\alpha$ ; TBS, Tris-buffered saline; TBST, TBS with Tween; TCA, tricarboxylic acid.

Inhibitory phosphorylation of PDC is catalyzed by members of a family of pyruvate dehydrogenase kinases (PDKs) (8,9). Four PDK isoforms have been identified, sequenced, and partially characterized (8). Gene (mRNA) expression of PDK1 and PDK2 has been detected in the pancreas (10), but the PDK isoforms specifically present in pancreatic islets have not been established. PDK activity is increased after prolonged (48 h) starvation, in a stable manner independent of the acute effects of metabolic effectors, in a range of oxidative tissues (11), including pancreatic islets (12). The PDK isoforms that contribute to increased PDK activity in islets after prolonged starvation remain to be identified. Prolonged (48 h) starvation increases the protein expression of PDK2 and PDK4 in liver (13,14) and kidney (14,15)—gluconeogenic tissues, which, like islets, contain high PC levels (16,17). The protein expression of PDK4 (but not of PDK2) is increased in heart (18) and oxidative skeletal muscle (19) in response to starvation. The effect of starvation to lead to selective accumulation of PDK4 protein in skeletal muscle is mimicked by long-term activation of peroxisome proliferator-activated receptor- $\alpha$  (PPAR- $\alpha$ ) by the selective agonist WY14,643 (20).

Abnormal lipid partitioning resulting in either depletion of islet lipid or accumulation of excessive islet lipid is potentially important in the development of pancreatic  $\beta$ -cell failure (21). PPARs are orphan nuclear receptors that are established fatty acid sensors, transducing changes in lipid supply into changes in gene expression (22). PPAR- $\alpha$  has been shown to control genes involved in lipid metabolism in islets and islet cell lines (23). PPAR- $\alpha$  mRNA expression is induced by high concentrations of fatty acids in rat islets (24). Conversely, islets or INS(832/13)  $\beta$ -cells exposed to high glucose show a 60–80% reduction in PPAR- $\alpha$  mRNA (23). It was suggested that a reduction in PPAR- $\alpha$  expression, together with an inappropriate rise in malonyl-CoA, might participate in the coordinated adaptation of  $\beta$ -cell glucose and lipid metabolism to hyperglycemia and be implicated in the mechanism of  $\beta$ -cell glucolipototoxicity (23).

In the present study, we delineated the effect of prolonged (48 h) starvation on the PDK isoform expression profile of rat pancreatic islets using antibodies against PDK1, PDK2, and PDK4 (no sufficiently specific antibodies are as yet available for use for Western blotting of PDK3). Because PPAR- $\alpha$  activation specifically upregulates PDK4 protein expression in skeletal muscle (20), we also determined whether PPAR- $\alpha$  activation with WY14,643 influences islet PDK isoform expression in the fed state, together with the potential influence of such modification on the regulation of insulin secretion by glucose and exogenous lipid.

## RESEARCH DESIGN AND METHODS

**Materials.** Organic solvents were of analytical grade and obtained from BDH (Poole, Dorset, U.K.). General laboratory reagents were from Roche Diagnostics (Lewes, East Sussex, U.K.) or from Sigma (Poole, Dorset, U.K.), with the following exceptions. Enhanced chemiluminescence (ECL) reagents, hyperfilm, and secondary antibodies were purchased from Amersham Pharmacia Biotech (Little Chalfont, Bucks, U.K.). Anti-PDK2 antibodies were generated in the authors' laboratory in rabbits against recombinant PDK2 (13). Anti-PDK1 and anti-PDK4 antibodies were generated in rabbits against individual recombinant proteins (8) and were provided by Prof. Bob Harris. Anti-PPAR- $\alpha$  antibody was purchased from Autogen Bioclear (Calne, Wiltshire, U.K.). Pirinixic acid (WY14,643) was purchased from Sigma. Bradford re-

agents were purchased from BioRad (Hemel Hempstead, Herts, U.K.). Kits for determination of plasma insulin, glucose, and nonesterified fatty acid (NEFA) concentrations were from Mercodia (Uppsala, Sweden), Roche Diagnostics, and Alpha Labs (Eastleigh, Hants, U.K.), respectively.

**Animals.** All studies were conducted in adherence to the regulations of the U.K. Animal Scientific Procedures Act (1986). Female albino Wistar rats (200–250 g) were purchased from Charles River (Margate, Kent, U.K.). Rats were maintained at a temperature of  $22 \pm 2^\circ\text{C}$  and subjected to a 12-h light/12-h dark cycle. Fed rats were given free access to standard, pelleted rodent diet purchased from Special Diet Services (Witham, Essex, U.K.; 52% carbohydrate, 15% protein, 3% lipid, and 30% nondigestible residue [by weight]; 2.61 kcal metabolizable energy/g). For determination of the effects of starvation, food was removed from cages at 48 h before the rats were killed. WY14,643 was administered to fed rats as a single intraperitoneal injection (50 mg/kg body wt), and rats were sampled after an additional 24 h. In all experiments, rats were allowed ad libitum access to water.

**Islet preparation and extraction.** Rats were anesthetized by injection of sodium pentobarbital (60 mg/ml in 0.9% NaCl; 1 ml/kg body wt i.p.); once locomotor activity had ceased, pancreases were excised, and islets were isolated by collagenase digestion (25). Islets (150–250, from a single individual animal) were extracted in ice-cold RIPA buffer (1% Triton, 1% sodium deoxycholate, 0.1% SDS, 0.15 mol/l NaCl, 0.01 mol/l sodium phosphate, 0.2 mmol/l phenylmethylsulfonyl fluoride, 10  $\mu\text{g/ml}$  leupeptin, 1.5 mg/ml benzamide, 50  $\mu\text{g/ml}$  aprotinin, 50  $\mu\text{g/ml}$  pepstatin A [in DMSO], pH 8.0). Total islet protein extracts were placed on ice for 20 min and centrifuged in an Eppendorf centrifuge (12,000g for 20 min at  $4^\circ\text{C}$ ), and the supernatants were stored ( $-20^\circ\text{C}$ ) until analysis. Protein concentrations were determined using the method of Bradford (26) using bovine serum albumin as standard. The assay was linear over the range of protein concentrations routinely used.

**Islet incubation.** Freshly prepared islets (up to 15) were placed into 1.5-ml Eppendorf tubes and, after preincubation (30 min at  $37^\circ\text{C}$  with Krebs-Ringer bicarbonate buffer [KRB]), incubated with 1,000  $\mu\text{l}$  of KRB containing 2 mmol/l or 20 mmol/l glucose in the presence or absence of triolein (1 mmol/l) for 60 min at  $37^\circ\text{C}$ . At the end of the incubation, aliquots of the medium were removed for the determination of insulin. Insulin release was linearly related to islet number (results not shown), but 10 islets from each preparation were routinely used for incubation. Incubations were in triplicate for each experimental condition.

**Immunoblotting.** Samples (50  $\mu\text{g}$  of total islet protein) were subjected to SDS-PAGE using a 12.5% resolving gel with a 6% stacking gel. After SDS-electrophoresis, resolved proteins were transferred electrophoretically to nitrocellulose membranes and then blocked for 2 h at room temperature with Tris-buffered saline (TBS) supplemented with 0.1% Tween (TBST) and 5% (wt/vol) nonfat powdered milk. The nitrocellulose blots were incubated for 2 h at room temperature with polyclonal antisera raised against specific recombinant PDK isoforms or PPAR- $\alpha$ , washed with 0.1% Tween in TBS ( $3 \times 10$  min), and incubated with the horseradish peroxidase-linked secondary antibody IgG anti-rabbit (1:2000, in 1% [wt/vol] nonfat milk in TBST) for 1 h at room temperature. Bound antibody was visualized using ECL according to the manufacturer's instructions. The blots were then exposed to Hyperfilm, and the signals were quantified by scanning densitometry and analyzed with Molecular Analyst 1.5 software (Biorad). For each representative immunoblot presented, the results are from a single gel exposed for a uniform duration, and each lane represents an islet preparation from a different rat.

**Analytical methods.** Plasma glucose concentrations were determined by a glucose oxidase method (Roche Diagnostic). Plasma immunoreactive insulin concentrations were measured by enzyme-linked immunosorbent assay (ELISA) using rat insulin as a standard (Mercodia). Plasma NEFA levels were determined with the Wako NEFA C test kit (Alpha Labs). Aliquots of blood sampled from the chest cavity were centrifuged for 20 min at 12,000g at  $4^\circ\text{C}$ , and plasma was stored at  $-20^\circ\text{C}$ .

**Statistical analysis.** Results are presented as the means  $\pm$  SE, with the numbers of rats in parentheses. Statistical analysis was performed by analysis of variance followed by Fisher's post hoc tests for individual comparisons or Student's *t* test as appropriate (Statview; Abacus Concepts, Berkeley, CA).  $P < 0.05$  was considered to be statistically significant.

## RESULTS

**Three PDK isoforms are expressed in pancreatic islets from fed rats.** Protein was extracted from batches of 150–250 islets, and protein expression of PDK isoforms 1, 2, and 4 were evaluated by immunoblotting with specific antibodies raised against each of these recombinant PDK proteins. (No sufficiently specific antibodies are as yet

TABLE 1  
Effects of starvation or activation of PPAR- $\alpha$  in vivo on plasma insulin and metabolite concentrations

	Control	Starvation (48 h)	WY14,643 treatment (24 h)
Insulin ( $\mu$ U/ml)	32 $\pm$ 4 (14)	11 $\pm$ 2* (9)	26 $\pm$ 5 (9)
Glucose (mmol/l)	8.8 $\pm$ 0.4 (8)	5.2 $\pm$ 0.4* (8)	8.5 $\pm$ 0.8 (6)
NEFA (mmol/l)	0.32 $\pm$ 0.04 (9)	1.56 $\pm$ 0.18* (9)	0.35 $\pm$ 0.04 (6)

Data are means  $\pm$  SE, with the numbers of rats in parentheses. Details of the protocols for starvation or treatment with WY14,643 are described in RESEARCH DESIGN AND METHODS. Plasma insulin, glucose, and NEFA concentrations in blood sampled from the chest cavity were determined using commercial kits. \* $P < 0.001$  versus control (fed ad libitum).

available for use for Western blotting of PDK3.) Western analysis using extracts of islets from fed rats detected protein expression of PDK1, PDK2, and PDK4. PDK1 was identified as an immunoreactive band running at 49 kDa, whereas PDK2 and PDK4 were detected as immunoreactive bands running at 46 kDa.

**Plasma substrate and insulin concentrations after starvation or PPAR- $\alpha$  activation in vivo.** Ambient plasma immunoreactive insulin concentrations fell markedly after starvation (Table 1). As expected, starvation for 48 h significantly increased plasma NEFA concentrations (by  $\sim$ 4.9-fold;  $P < 0.001$ ), whereas plasma glucose concentrations declined (by 41%;  $P < 0.001$ ) compared with control rats (fed ad libitum on standard diet) (Table 1). WY14,643 treatment of fed rats for 24 h in vivo did not significantly influence plasma insulin, glucose, or NEFA concentrations (Table 1).

**Prolonged starvation modulates the protein expression of all three PDK isoforms in rat pancreatic islets.** PDK1, PDK2, and PDK4 protein expression was also detected in pancreatic islets from rats that were starved for 48 h (Fig. 1). Islet PDK1 protein expression was decreased by 48-h starvation (by 27%;  $P < 0.01$ ). Similarly, islet protein expression of PDK2, the "ubiquitous" PDK isoform, was also downregulated (by 10%; NS). In marked contrast, we observed a substantial  $\sim$ 2.3-fold increase ( $P < 0.01$ ) in the amount of PDK4 protein expressed in rat pancreatic islets in response to 48-h starvation (Fig. 1). Thus, the amount of PDK4 protein relative to total PDK protein (PDK1 + PDK2 + PDK4) in islets is greatly increased after prolonged starvation.

**Exposure to WY14,643 selectively upregulates PDK4 but does not alter PDK1 or PDK2 isoform protein expression in rat pancreatic islets.** Exposure of fed

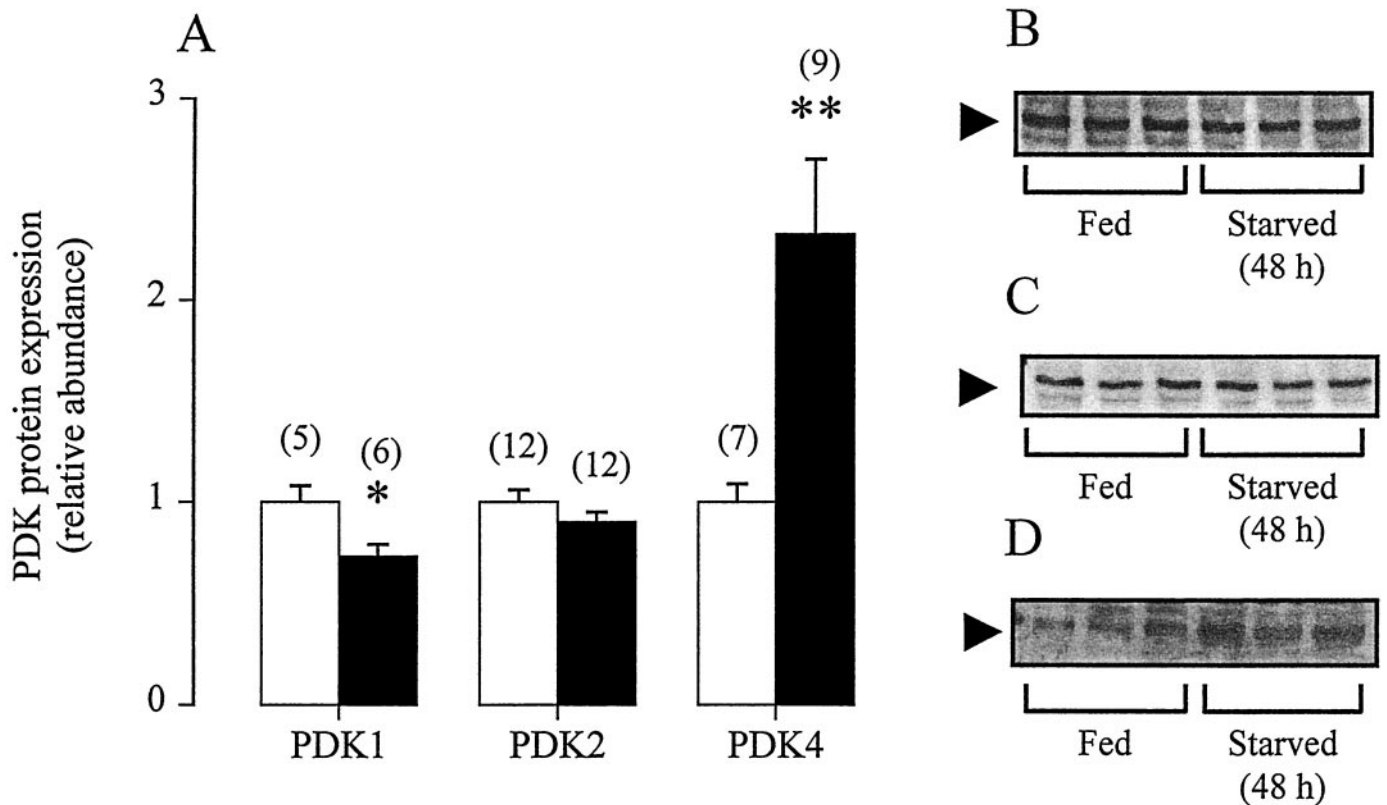
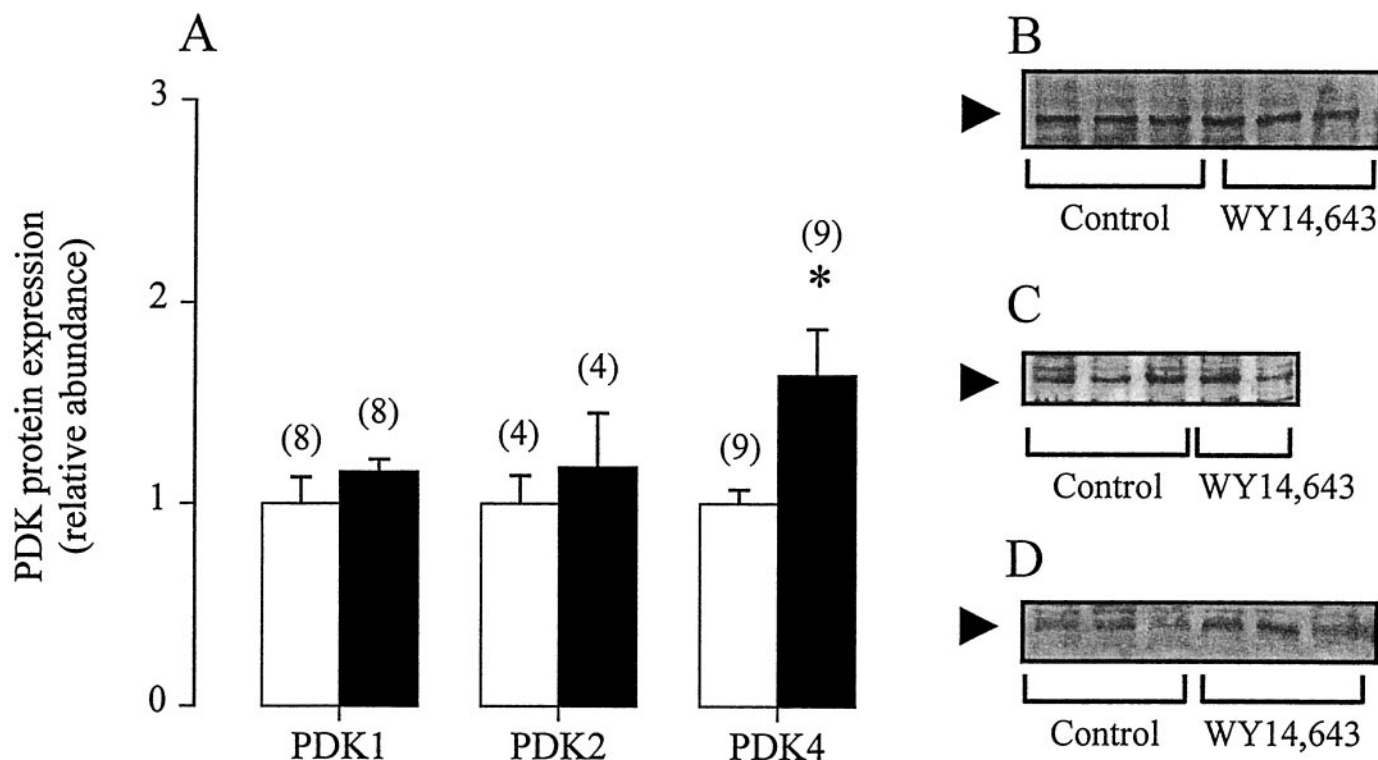


FIG. 1. Effects of 48-h starvation on PDK protein expression in isolated islets. Rabbit polyclonal antisera raised against PDK1, PDK2, and PDK4 were used to detect PDK protein by Western blot analysis. A: Quantification of Western blot analysis of PDK isoform protein expression. Western blots were analyzed by scanning densitometry using Molecular Analyst 1.5 software. Data are means  $\pm$  SE for the number of islet preparations from individual rats in each experimental group, as indicated in parentheses above the bar. Typical immunoblots of PDK1 (B), PDK2 (C), and PDK4 (D) protein expression are shown for individual islet preparations from three control fed versus three starved rats. \* $P < 0.05$  versus control; \*\* $P < 0.01$  versus control.



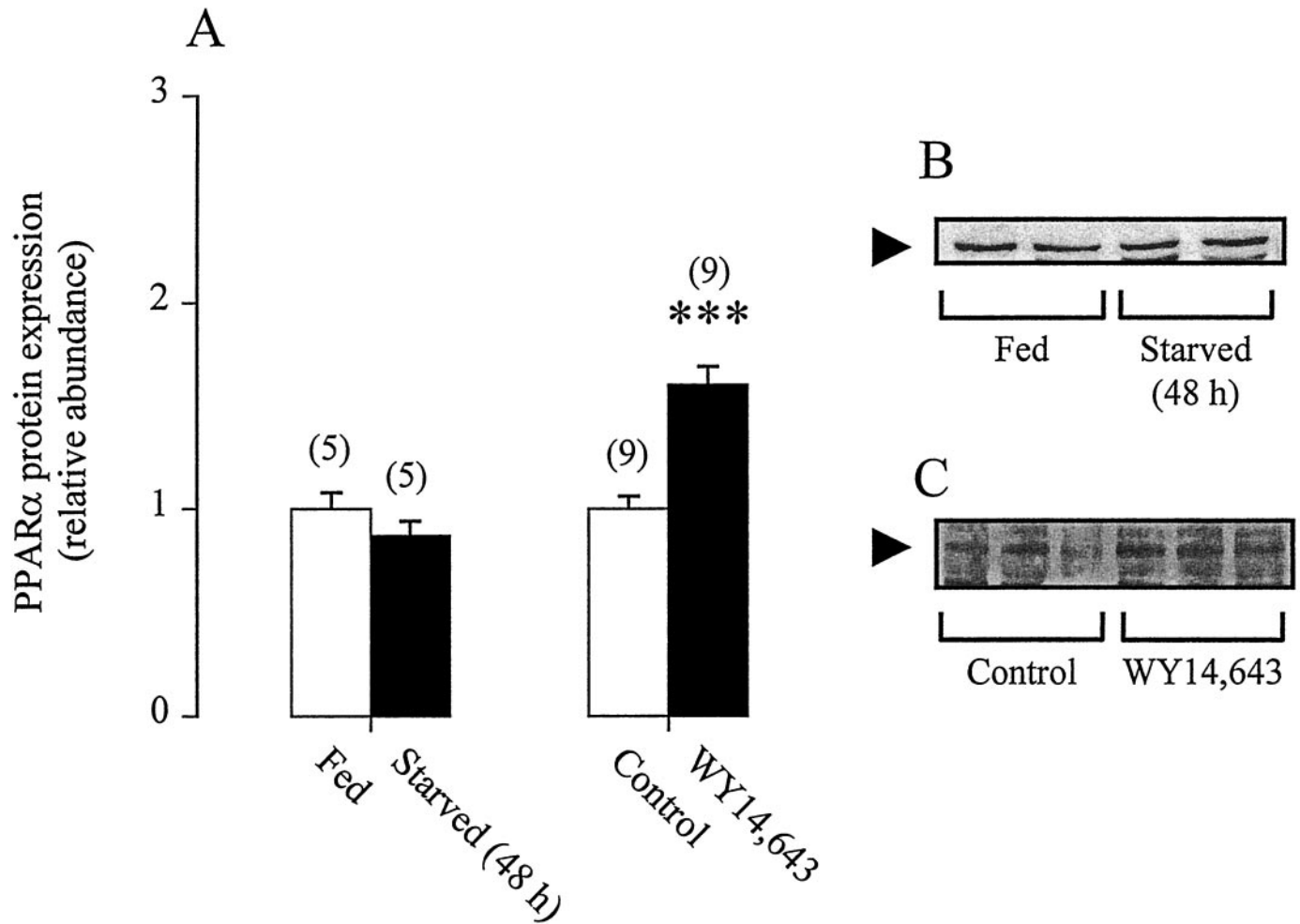
**FIG. 2.** Effects of activation of PPAR- $\alpha$  in vivo (24 h) on PDK protein expression in isolated islets. Details of treatment with WY14,643 are described in RESEARCH DESIGN AND METHODS. Rabbit polyclonal antisera raised against PDK1, PDK2, and PDK4 were used to detect PDK protein with Western blot analysis. **A:** Quantification of Western analysis of PDK isoform protein expression. Western blots were analyzed by scanning densitometry using Molecular Analyst 1.5 software. Data are means  $\pm$  SE for the number of islet preparations from individual rats in each experimental group, as indicated in parentheses above the bar. Typical immunoblots of PDK1 (**B**), PDK2 (**C**), and PDK4 (**D**) protein expression are shown for three individual islet preparations from control fed rats versus two (PDK2) or three (PDK1 and PDK4) WY14,643-treated fed rats. \* $P < 0.05$  versus control.

rats to the PPAR- $\alpha$  agonist WY14,643 for 24 h in vivo led to a substantial  $\sim 1.8$ -fold increase ( $P < 0.01$ ) in the amount of PDK4 protein expressed in rat pancreatic islets (Fig. 2). In contrast, WY14,643 administration did not significantly affect protein expression of either PDK1 or PDK2 (Fig. 2). The effects of WY14,643 administration on PDK4 protein expression were not a consequence of decreased caloric intake (control intake  $70 \pm 10$  kcal/day; WY14,643-treated intake  $64 \pm 3$  kcal/day).

**Exposure to WY14,643 in vivo increases PPAR- $\alpha$  protein expression in rat pancreatic islets.** It was demonstrated previously that fasting induces PPAR- $\alpha$  mRNA expression in liver (27), whereas PPAR- $\alpha$  expression is markedly reduced in the fat-laden islets of obese, prediabetic Zucker diabetic (*fa/fa*) rats (24). We analyzed the potential role of changes in PPAR- $\alpha$  activation in the regulation of PPAR- $\alpha$  protein expression in the islet (Fig. 3). Islet PPAR- $\alpha$  was identified as an immunoreactive band running at 52 kDa in islets from control, fed rats. Exposure to WY14,643 in vivo led to a significant 1.6-fold increase ( $P < 0.001$ ) in islet PPAR- $\alpha$  protein expression. This finding consolidates previous studies showing that culture of normal islets with the PPAR- $\alpha$  ligand clofibrate increases PPAR- $\alpha$  mRNA expression in normal islets but not in islets from *fa/fa* rats (24). In contrast, we failed to observe any modification of islet PPAR- $\alpha$  protein expression after 48 h of starvation (Fig. 3).

**Starvation and PPAR- $\alpha$  activation in vivo modulate the characteristics of glucose-stimulated insulin se-**

**cretion by isolated pancreatic islets.** We evaluated the possible effects of the altered PDK profile and/or PPAR- $\alpha$  expression observed in response to starvation or exposure to WY14,643 in vivo on glucose-stimulated insulin secretion (GSIS) using isolated islets. Comparison of the insulin secretory responses to glucose and triolein was made with islets from control fed rats. Table 2 shows the results obtained using batch incubation of 10 rat islets incubated for 1 h with 20 mmol/l glucose in the absence or presence of exogenous triglyceride (1 mmol/l triolein). GSIS was modestly but significantly impaired in islets from fed rats on incubation with triolein ( $\sim 20\%$  inhibition;  $P < 0.05$ ). Starvation (48 h), which modestly decreased islet PDK1 and PDK2 protein expression but greatly increased islet PDK4 protein expression, significantly impaired GSIS in the absence of triolein (by 57%;  $P < 0.001$ ). However, the further addition of triolein significantly increased GSIS with islets from starved (c.f. fed) rats by 64% ( $P < 0.05$ ). As a result, GSIS in the presence of triolein did not differ significantly between islets prepared from fed rats or rats that were starved for 48 h. GSIS by islets prepared from WY14,643-treated fed rats, in which PDK4 was specifically upregulated, did not differ significantly from that seen with islets from control fed rats. Thus, upregulation of islet fatty acid oxidation capacity as a consequence of previous upregulation of PPAR- $\alpha$  in vivo (which might be predicted to diminish islet TAG content in vivo) had little effect on GSIS on subsequent incubation with triolein. Importantly, the response to triolein addition (a trend toward suppres-



**FIG. 3.** Effects of 48-h starvation or activation of PPAR- $\alpha$  in vivo on PPAR- $\alpha$  protein expression in isolated islets. Details of treatment with WY14,643 are described in RESEARCH DESIGN AND METHODS. Rabbit polyclonal antisera raised against PPAR- $\alpha$  were used to detect PPAR- $\alpha$  protein with Western blot analysis. Quantification of Western blot analysis of PPAR- $\alpha$  isoform expression are shown in *A* together with typical immunoblots for individual islet preparations from three control fed versus three starved rats (*B*) and three control versus three WY14,643-treated fed rats (*C*). Western blots were analyzed by scanning densitometry using Molecular Analyst 1.5 software. Data are means  $\pm$  SE for the number of islet preparations from individual rats in each experimental group, as indicated in parentheses above the bar. \*\*\* $P < 0.001$  versus control.

sion of GSIS) observed with islets prepared from fed, WY14,643-treated rats more closely resembled that of islets prepared from fed rats than of islets prepared from starved rats.

#### PPAR- $\alpha$ activation but not starvation increases insulin secretion at a nonstimulatory glucose concentra-

tion. Because prolonged exposure to fatty acids decreases the threshold for the insulin secretory response to glucose (28), we compared the effects of starvation and PPAR- $\alpha$  activation in vivo on the response to 2 mmol/l glucose alone or in combination with 1 mmol/l triolein (Table 2). Starvation (48 h) did not affect basal insulin secretion at 2

**TABLE 2**

Effect of antecedent starvation or activation of PPAR- $\alpha$  in vivo on GSIS in islets incubated in the absence or presence of exogenous triglyceride

Group	Insulin secretion ( $\mu$ U/10 islets per h)			
	Glucose (2 mmol/l)	Glucose (2 mmol/l) plus triolein (1 mmol/l)	Glucose (20 mmol/l)	Glucose (20 mmol/l) plus triolein (1 mmol/l)
Control (fed ad libitum)	118 $\pm$ 20 (9)	90 $\pm$ 10 (10)	1,024 $\pm$ 76 (14)	828 $\pm$ 115§ (10)
Starved (48 h)	132 $\pm$ 22 (13)	106 $\pm$ 10 (10)	440 $\pm$ 31‡ (11)	722 $\pm$ 97§ (9)
WY14,643-treated (24 h)	293 $\pm$ 51* (9)	200 $\pm$ 40† (8)	1,002 $\pm$ 117 (10)	808 $\pm$ 58 (7)

Data are means  $\pm$  SE, with the numbers of rats in parentheses. Freshly prepared islets from each experimental group were incubated with either 2 or 20 mmol/l glucose in the presence or absence of triolein (1 mmol/l) for 60 min at 37°C. At the end of the incubation, aliquots of media were removed for the insulin determination. Incubations were in triplicate for each experimental condition. \* $P < 0.01$  versus control; † $P < 0.05$  versus control; ‡ $P < 0.001$  versus control; § $P < 0.05$  versus no triolein addition.

mmol/l glucose. By contrast, exposure to WY14,643 *in vivo* greatly enhanced insulin secretion at 2 mmol/l glucose (by 2.5 fold;  $P < 0.01$ ). The addition of triolein did not depress insulin secretion at 2 mmol/l glucose with islets from fed rats or enhance insulin secretion at 2 mmol/l glucose with islets from starved rats. A trend toward lower insulin secretion at 2 mmol/l glucose was observed with islets from fed, WY14,643-treated rats but, as at 20 mmol/l glucose, this trend did not achieve significance.

## DISCUSSION

In the present study, we demonstrated the protein expression of three PDK isoforms (PDK1, PDK2, and PDK4) in rat pancreatic islets (no sufficiently specific antibodies are as yet available for PDK3). We also showed, for the first time, that the PDK isoform protein expression profile of rat pancreatic islets is selectively modified in response to prolonged starvation. A major novel finding is that PDK1 and (to a lesser extent) PDK2 protein expression in the rat pancreatic islet is suppressed by prolonged starvation, whereas the protein expression of the third PDK isoform, PDK4, is specifically upregulated by starvation. We also demonstrated, for the first time, specific upregulation of PDK4 and PPAR- $\alpha$  protein expression in rat pancreatic islets in response to the administration of the PPAR- $\alpha$  agonist WY14,643, identifying a potential role for PPAR- $\alpha$ -linked functions in the islet response to starvation. We analyzed the impact of antecedent changes in islet PDK protein expression and PPAR- $\alpha$  activation *in vivo* on insulin secretion using isolated islets. We demonstrated that upregulation of islet PDK4 expression is associated with impaired GSIS in islets from starved rats but not in islets from WY14,643-treated fed rats, arguing that the major impairment in GSIS introduced by starvation is at a site other than PDC. By contrast, we demonstrated that activation of PPAR- $\alpha$  *in vivo* for 24 h leads to a lowered threshold for GSIS, mimicking that observed in response to prolonged exposure to fatty acids *in vitro* (28). Such an effect might underlie relative hyperinsulinemia in insulin-resistant states associated with an elevated fatty acid supply. We propose that this may be a consequence of a long-term stable modification of islet function elicited *in vivo* in response to sustained PPAR- $\alpha$  activation, but increased islet PDK4 protein expression does not solely mediate this response.

Acute exposure of islets to fatty acids only modestly influences glucose oxidation in islets from fed rodents (12). Moreover, concentrations of glucose that are stimulatory for insulin secretion suppress fatty acid  $\beta$ -oxidation (29–31), a result consistent with effects of glucose to elevate malonyl-CoA concentrations via flux through PDC. A relatively refractory insulin secretory response to the further addition of exogenous lipid (triolein) was demonstrated in the present study with islets from fed rats incubated with 20 mmol/l glucose. Our results are consistent with the hypothesis that either incoming fatty acids are predominantly esterified rather than oxidized in the fed state or both glucose and fat can be oxidized simultaneously by the islet. The presence of PDK1 protein expression in rat islets is remarkable as, hitherto, PDK1 protein expression has been thought to be relatively specific to the heart (8). Differences among PDK1, PDK2, and PDK4 with

respect to their acute regulation by metabolites have been identified using recombinant proteins *in vitro* (8). The major difference between rPDK1 and rPDK4, both of which are PDK isoforms of relatively high specific activity, is that rPDK1 activity is not greatly stimulated by an increased NADH/NAD<sup>+</sup> concentration ratio unless acetyl-CoA concentrations are also elevated. By contrast, rPDK4 is activated by elevated NADH/NAD<sup>+</sup> concentration ratio, but the further addition of acetyl-CoA has little additional effect. The behavior of rPDK2 resembles rPDK1 with respect to regulation by NADH/NAD<sup>+</sup> and acetyl-CoA, but it has lower specific activity. A high functional contribution of PDK1 (a high-specific activity PDK isoform) to total PDK activity in the fed state would be predicted to confer a degree of insensitivity of PDK activity (and therefore PDC activity) to altered mitochondrial NADH/NAD<sup>+</sup> ratios. This would be predicted to allow acetyl-CoA and citrate production from glucose even when concomitant fatty acid oxidation elevates intramitochondrial NADH/NAD<sup>+</sup> concentration ratios. We therefore propose that the presence of the PDK1 isoform may be important in facilitating the concomitant use of glucose and lipid by the pancreatic islet.

Although acute exposure of islets to fatty acids only modestly influences glucose oxidation in islets from fed rodents (12), islet PDC activity is suppressed after prolonged starvation. This effect is observed in conjunction with increased islet total PDK activity and impaired GSIS (12). In previous studies by Randle and colleagues (32), an effect of starvation to modify overall islet PDK content could not be detected when PDK content was assayed by ELISA with antibodies raised against PDK  $\alpha$ -chain. Armed with a current, more detailed understanding of the PDK system, together with specific antibodies to individual PDK isoforms, we demonstrated, for the first time, that the starvation-induced increase in islet PDK activity is associated with selective upregulation of the protein expression of a single PDK isoform, PDK4. This selective upregulation of PDK4 protein expression is analogous to that observed in heart and oxidative skeletal muscle (18,19). It follows, therefore, that PDK4 upregulation in the islet is likely to underlie the stable increase in PDK activity observed in response to starvation. The previous failure to detect any overall change in PDK  $\alpha$ -chain protein expression (32) possibly reflects the accompanying suppression of the expression of the other two PDK isoforms found in the pancreatic islet (present study). The ~30% suppression of PDK1 and PDK2 protein expression observed in starvation may be a mechanism to blunt the response of PDK activity to a rise in acetyl-CoA. This would ensure that islet glucose oxidation and, therefore, energy metabolism are not compromised when a fast is terminated and glucose reenters the system. Such a concept is supported by the effect of exogenous triglyceride to enhance GSIS with islets from starved rats (present experiments) and by studies *in vivo* demonstrating that elevated circulating fatty acids are permissive for GSIS after fasting (33; rev. in 34).

An increased [PDK4/(PDK1 + PDK2)] protein expression ratio in muscle in starvation correlates closely with lesser sensitivity of PDK activity to suppression by pyruvate (19,35). By analogy, the marked upregulation of islet PDK4 protein expression observed in conjunction with

modest suppression of islet PDK1/PDK2 protein expression in response to starvation would be predicted to attenuate effects of an increased pyruvate supply to activate islet PDC. Under such conditions, concomitant elevation of acetyl-CoA concentrations and NADH/NAD<sup>+</sup> concentration ratios will activate all three islet PDK isoforms, and acetyl-CoA will simultaneously activate PC. Thus, the PDK isoform profile found in the islet after starvation—relatively insensitive to mitochondrial pyruvate accumulation but sensitive, by virtue of PDK4 upregulation, to a rise in mitochondrial NADH levels—would be predicted to favor anaplerotic entry of pyruvate into the tricarboxylic acid (TCA) cycle via pyruvate carboxylation, even if pyruvate levels were to increase rapidly (e.g., because of stimulation of glycolytic flux). Under these circumstances, islet energy metabolism would be reliant on fatty acid  $\beta$ -oxidation for acetyl-CoA production and ATP generation. Furthermore, increased glycolysis (e.g., in response to an acute rise in glucose supply) would, by virtue of suppression of acetyl-CoA production via PDC, be unable to elicit stimulation of insulin secretion through citrate accumulation unless adequate flux from fatty acids to acetyl-CoA is sustained.

It has been hypothesized that entry of pyruvate into the TCA cycle via nonoxidative pathways is an important component of fuel signaling for stimulation of insulin secretion (5). We propose that the specific upregulation of PDK4 protein expression in the pancreatic islet in starvation, by maintaining PDC activity at relatively low levels, despite decreased protein expression of both PDK1 and PDK2, functions to facilitate pyruvate carboxylation to oxaloacetate (rather than oxidative decarboxylation via PDC). This is predicted to facilitate entry of acetyl-CoA derived from fatty acid  $\beta$ -oxidation into the TCA cycle via citrate formation. Our results therefore suggest that PDK4 is a vital component of the mechanism by which the pancreatic  $\beta$ -cell senses lipid abundance and, in addition, support the recently proposed role of citrate as a signal of islet fuel supply (5). We also propose that upregulation of PDK4 protein expression in islets may, in part, account for the observation that circulating fatty acids are essential for efficient GSIS after prolonged fasting (36).

We addressed the question as to the mechanism(s) by which modulation of the islet PDK4 protein expression profile might be achieved. PPAR- $\alpha$ , a member of the ligand-activated nuclear receptor superfamily, is an essential transcriptional regulator of a large number of genes involved in lipid metabolism, especially those concerned with peroxisomal and mitochondrial fatty acid  $\beta$ -oxidation (22). Activation of PPAR- $\alpha$  by the specific agonist WY14,643 in vivo upregulated islet PDK4 protein expression within 24 h (i.e., within the time scale of the effect of starvation to upregulate islet PDK4 protein expression). Our data are therefore consistent with the concept that activation of islet PPAR- $\alpha$  by an endogenous natural ligand is an important component of the overall response of islet PDK4 protein expression to starvation. By contrast, our data clearly indicate that downregulation of islet PDK1 protein expression in starvation is not achieved via activation of PPAR- $\alpha$ . Insulin secretion by the pancreatic  $\beta$ -cell is tightly coupled to prevailing blood glucose levels via changes in glucose metabolism initiated via glucose

transporter GLUT2 and glucokinase, both of which have a relatively high  $K_m$  for glucose. Islets also contain hexokinase (37), which has a relatively low  $K_m$  for glucose, but hexokinase is not thought to participate in the islet glucose-sensing mechanism (38,39). It is presumed that in the WY14,643-treated fed group, there is adequate “glucose sensing” and metabolism via GLUT2 and glucokinase to permit normal GSIS in the absence of triolein. Because the putative upregulation of the islet capacity for fatty acid oxidation by WY14,643 treatment in vivo does not markedly influence GSIS, it seems likely that fatty acid oxidation is suppressed proximal to  $\beta$ -oxidation. The lack of influence of PDK4 upregulation on GSIS with islets from fed rats is also consistent with the concept that islet fatty acid oxidation is relatively suppressed in the fed state.

#### ACKNOWLEDGMENTS

This study was supported in part by project grants from Diabetes UK (formerly the British Diabetic Association) (RD98/1625 and RD01/2249) to M.C.S. and M.J.H.

#### REFERENCES

- MacDonald MJ: Glucose enters mitochondrial metabolism via both carboxylation and decarboxylation of pyruvate in pancreatic islets. *Metabolism* 42:1229–1231, 1993
- Khan A, Ling ZC, Landau BR: Quantifying the carboxylation of pyruvate in pancreatic islets. *J Biol Chem* 271:2539–2542, 1996
- Schuit F, De Vos A, Farfari S, Moens K, Pipeleers D, Brun T, Prentki M: Metabolic fate of glucose in purified islet cells. Glucose-regulated anaplerosis in beta cells. *J Biol Chem* 272:18572–18579, 1997
- Sekine N, Cirulli V, Regazzi R, Brown LJ, Gine E, Tamarit-Rodriguez J, Girotti M, Marie S, MacDonald MJ, Wollheim CB, Saffitz JE, Rutter GA: Low lactate dehydrogenase and high mitochondrial glycerol phosphate dehydrogenase in pancreatic beta-cells: potential role in nutrient sensing. *J Biol Chem* 269:4895–4902, 1994
- Farfari S, Schulz V, Corkey B, Prentki M: Glucose-regulated anaplerosis and cataplerosis in pancreatic beta-cells: possible implication of a pyruvate/citrate shuttle in insulin secretion. *Diabetes* 49:718–726, 2000
- Prentki M, Vischer S, Glennon MC, Regazzi R, Deeney JT, Corkey BE: Malonyl-CoA and long chain acyl-CoA esters as metabolic coupling factors in nutrient-induced insulin secretion. *J Biol Chem* 267:5802–5810, 1992
- Prentki M, Corkey BE: Are the beta-cell signaling molecules malonyl-CoA and cystolic long-chain acyl-CoA implicated in multiple tissue defects of obesity and NIDDM? *Diabetes* 45:273–283, 1996
- Bowker-Kinley MM, Davis WI, Wu P, Harris RA, Popov KM: Evidence for existence of tissue-specific regulation of the mammalian pyruvate dehydrogenase complex. *Biochem J* 329:191–196, 1998
- Popov KM, Hawes JW, Harris RA: Mitochondrial alpha-ketoacid dehydrogenase kinases: a new family of protein kinases. *Adv Second Messenger Phosphoprotein Res* 31:105–111, 1997
- Gudi R, Bowker-Kinley MM, Kedishvili NY, Zhao Y, Popov KM: Diversity of the pyruvate dehydrogenase kinase gene family in humans. *J Biol Chem* 270:28989–28994, 1995
- Randle PJ, Priestman DA, Mistry S, Halsall A: Mechanisms modifying glucose oxidation in diabetes mellitus. *Diabetologia* 37 (Suppl. 2):S155–S161, 1994
- Zhou YP, Priestman DA, Randle PJ, Grill VE: Fasting and decreased B cell sensitivity: important role for fatty acid-induced inhibition of PDH activity. *Am J Physiol Endocrinol Metab* 270:E988–E994, 1996
- Sugden MC, Fryer LG, Orfali KA, Priestman DA, Donald E, Holness MJ: Studies of the long-term regulation of hepatic pyruvate dehydrogenase kinase. *Biochem J* 329:89–94, 1998
- Wu P, Blair PV, Sato J, Jaskiewicz J, Popov KM, Harris RA: Starvation increases the amount of pyruvate dehydrogenase kinase in several mammalian tissues. *Arch Biochem Biophys* 381:1–7, 2000
- Sugden MC, Holness MJ, Donald E, Lall H: Substrate interactions in the acute and long-term regulation of renal glucose oxidation. *Metabolism* 48:707–715, 1999
- MacDonald MJ: Influence of glucose on pyruvate carboxylase expression in pancreatic islets. *Arch Biochem Biophys* 319:128–132, 1995

17. Jitrapakdee S, Wallace JC: Structure, function and regulation of pyruvate carboxylase. *Biochem J* 340:1–16, 1999
18. Wu P, Sato J, Zhao Y, Jaskiewicz J, Popov KM, Harris RA: Starvation and diabetes increase the amount of pyruvate dehydrogenase kinase isoenzyme 4 in rat heart. *Biochem J* 329:197–201, 1998
19. Sugden MC, Kraus A, Harris RA, Holness MJ: Fibre-type specific modification of the activity and regulation of skeletal muscle pyruvate dehydrogenase kinase (PDK) by prolonged starvation and refeeding is associated with targeted regulation of PDK isoenzyme 4 expression. *Biochem J* 346:651–657, 2000
20. Wu P, Inskip K, Bowker-Kinley MM, Popov KM, Harris RA: Mechanism responsible for inactivation of skeletal muscle pyruvate dehydrogenase complex in starvation and diabetes. *Diabetes* 48:1593–1599, 1999
21. Zhou YT, Shimabukuro M, Lee Y, Koyama K, Higa M, Ferguson T, Unger RH: Enhanced de novo lipogenesis in the leptin-unresponsive pancreatic islets of prediabetic Zucker diabetic fatty rats: role in the pathogenesis of lipotoxic diabetes. *Diabetes* 47:1904–1908, 1998
22. Kersten S, Desvergne B, Wahli W: Roles of PPARs in health and disease. *Nature* 405:421–424, 2000
23. Roduit R, Morin J, Masse F, Segall L, Roche E, Newgard CB, Assimakopoulos-Jeannot F, Prentki M: Glucose down-regulates the expression of the peroxisome proliferator-activated receptor-alpha gene in the pancreatic beta-cell. *J Biol Chem* 275:35799–35806, 2000
24. Zhou YT, Shimabukuro M, Wang MY, Lee Y, Higa M, Milburn JL, Newgard CB, Unger RH: Role of peroxisome proliferator-activated receptor alpha in disease of pancreatic beta cells. *Proc Natl Acad Sci U S A* 95:8898–8903, 1998
25. Lacy PE, Kostianovsky M: Method for the isolation of intact islets of Langerhans from the rat pancreas. *Diabetes* 16:35–39, 1967
26. Bradford MM: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72:248–254, 1976
27. Kersten S, Seydoux J, Peters JM, Gonzalez FJ, Desvergne B, Wahli W: Peroxisome proliferator-activated receptor alpha mediates the adaptive response to fasting. *J Clin Invest* 103:1489–1498, 1999
28. Hosokawa H, Corkey BE, Leahy JL: Beta-cell hypersensitivity to glucose following 24-h exposure of rat islets to fatty acids. *Diabetologia* 40:392–397, 1997
29. Berne C: The metabolism of lipids in mouse pancreatic islets. The oxidation of fatty acids and ketone bodies. *Biochem J* 152:661–666, 1975
30. Vara E, Tamarit-Rodriguez J: Glucose stimulation of insulin secretion in islets of fed and starved rats and its dependence on lipid metabolism. *Metabolism* 35:266–271, 1986
31. Tamarit-Rodriguez J, Vara E, Tamarit J: Starvation-induced changes of palmitate metabolism and insulin secretion in isolated rat islets stimulated by glucose. *Biochem J* 221:317–324, 1984
32. Priestman DA, Mistry SC, Halsall A, Randle PJ: Role of protein synthesis and of fatty acid metabolism in the longer-term regulation of pyruvate dehydrogenase kinase. *Biochem J* 300:659–664, 1994
33. Stein DT, Esser V, Stevenson BE, Lane KE, Whiteside JH, Daniels MB, Chen S, McGarry JD: Essentiality of circulating fatty acids for glucose-stimulated insulin secretion in the fasted rat. *J Clin Invest* 97:2728–2735, 1996
34. McGarry JD, Dobbins RL: Fatty acids, lipotoxicity and insulin secretion. *Diabetologia* 42:128–138, 1999
35. Priestman DA, Orfali KA, Sugden MC: Pyruvate inhibition of pyruvate dehydrogenase kinase: effects of progressive starvation and hyperthyroidism in vivo, and of dibutyl cyclic AMP and fatty acids in cultured cardiac myocytes. *FEBS Lett* 393:174–178, 1996
36. Dobbins RL, Chester MW, Daniels MB, McGarry JD, Stein DT: Circulating fatty acids are essential for efficient glucose-stimulated insulin secretion after prolonged fasting in humans. *Diabetes* 47:1613–1618, 1998
37. Heimberg H, De Vos A, Vandercammen A, Van Schaftingen E, Pipeleers D, Schuit F: Heterogeneity in glucose sensitivity among pancreatic beta-cells is correlated to differences in glucose phosphorylation rather than glucose transport. *EMBO J* 12:2873–2879, 1993
38. Meglasson MD, Matschinsky FM: Pancreatic islet glucose metabolism and regulation of insulin secretion. *Diabetes Metab Rev* 2:163–214, 1986
39. Giroix MH, Sener A, Pipeleers DG, Malaisse WJ: Hexose metabolism in pancreatic islets: inhibition of hexokinase. *Biochem J* 223:447–453, 1984