Protein Feeding Promotes Redistribution of Endogenous Glucose Production to the Kidney and Potentiates Its Suppression by Insulin

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The aim of this study was to assess in rats the effect of protein feeding on the: 1) distribution of endogenous glucose production (EGP) among gluconeogenic organs, and 2) repercussion on the insulin sensitivity of glucose metabolism. We used gene expression analyses, a combination of glucose tracer dilution and arteriovenous balance to quantify specific organ release, and hyperinsulinemic euglycemic clamps to assess EGP and glucose uptake. Protein feeding promoted a dramatic induction of the main regulatory gluconeogenic genes (glucose-6 phosphatase and phosphoenolpyruvate carboxykinase) in the kidney, but not in the liver. As a consequence, the kidney glucose release was markedly increased, compared with rats fed a normal starch diet. Protein feeding ameliorated the suppression of EGP by insulin and the sparing of glycogen storage in the liver but had no effect on glucose uptake. Combined with the previously reported induction of gluconeogenesis in the small intestine, the present work strongly suggests that a redistribution of glucose production among gluconeogenic organs might occur upon protein feeding. This phenomenon is in keeping with the improvement of insulin sensitivity of EGP, most likely involving the hepatic site. These data shed a new light on the improvement of glucose tolerance, previously observed upon increasing the amount of protein in the diet, in type 2 diabetic patients. (*Endocrinology* 150: 616–624, 2009)

Insulin resistance is the hallmark of the so-called metabolic syndrome and of its associated pathology type 2 diabetes. Among various altered metabolic functions, increased endogenous glucose production (EGP) has long been recognized to be a crucial step in the metabolic staging of the illness from insulin resistance toward impaired glucose tolerance and further to diabetes (1, 2). Until recently it was considered that only the liver and kidney are capable of releasing glucose in blood because both are the only organs considered to express glucose-6 phosphatase (Glc6Pase), the key enzyme of glucose production. In contradiction to this dogma, we demonstrated that the small intestine (SI) is a third gluconeogenic organ, expressing Glc6Pase and other genes required for gluconeogenesis, and is able to contribute to EGP in the fasting situation and insulinopenic diabetes (3–6) (for review, see Ref. 7).

As a first analysis, one may suppose that the induction of glucose production in any of the three gluconeogenic organs, in the postabsorptive state, should result in a global augmentation of EGP, thereby favoring a possible deleterious action on glucose homeostasis. However, previous data from our group have suggested that the reality is probably more complex. For instance, in insulin-resistant high-fat fed rats, we reported that the beneficial decreasing effect of the antidiabetic agent metformin on EGP takes place despite the concomitant appearance of a small component of glucose production by the intestine (8). Furthermore, the induction of gluconeogenesis in the SI of rats fed a protein-enriched diet (PED) does not correlate with any increase of EGP in these animals (9). This suggests that a compensatory decrease of the contribution of either the liver or the kidney to glucose production should occur in these situations. Actually, a decrease of glucose production principally taking place in the liver might be favored because of the well-known inhibitory effect of portal glucose on hepatic glucose release (10).

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Abbreviations: EGP, Endogenous glucose production; FX, fractional extraction; Glc6Pase, glucose-6 phosphatase; IGP, intestinal glucose production; PED, protein-enriched diet; PEPCK, cytosolic phosphoenolpyruvate; PEPCK-c, cytosolic phosphoenolpyruvate carboxykinase; RBF, renal blood flow; RGB, renal glucose balance; RGR, renal glucose release; RGU, renal glucose uptake; SA, specific activity; SED, starch-enriched diet; SI, small intestine.

High-protein feeding represents a special nutritional situation, deserving further characterization. It is well known that food protein ingestion may play an indirect beneficial role in glucose homeostasis, via the induction of satiety effects and weight loss in both animals and humans (11). We recently reported that the induction of intestinal gluconeogenesis, by way of the portal sensing of intestinal glucose released, and activation of the hypothalamic areas regulating energy homeostasis, is a mechanism by which food proteins induce the diminution of food intake in rats (9). Moreover, recent results also indicate that the parameters of glucose tolerance, including the level of glycated hemoglobin, are markedly improved in the short-term in type 2 diabetic patients after increasing the proportion of dietary proteins for a few weeks, independently of any effect on body weight (12, 13) (for review, see Ref. 14). However, over the long term, it is considered that the improvement of insulin sensitivity in diabetic patients is the result of weight loss (11). Given the importance of EGP in glucose homeostasis (1, 2), and possibly in the initiation of type 2 diabetes, we raised the question of the effect of protein feeding on both liver and kidney gluconeogenesis. To this aim we used gene expression analysis to evaluate whether protein act as a regulatory signal on gluconeogenic genes in both tissues, and a combination of glucose tracer dilution and arteriovenous glucose balance determination to evaluate whether this translates in glucose release by the targeted tissues (5, 9). It must be noted that gluconeogenesis may proceed from different precursors, *e.g.* alanine, lactate, glutamine, or glycerol, the utilization of which may vary according to the organ considered. Specific gluconeogenic pathway from these substrates could be quantified using relevant carbon-labeled precursors (5, 15). The question addressed herein (repartition of EGP among gluconeogenic organs) makes it relevant to use the tritiated glucose approach. Indeed, the latter allows one to quantify the whole glucose release from the organ considered, regardless of the precursor. In combination with these studies, we addressed the question of the effect of protein feeding on insulin sensitivity of both EGP and total glucose uptake, using the classical hyperinsulinemic euglycemic clamp approach (16).

Materials and Methods

Animals and diets

Seven-week-old male Sprague Dawley rats (Charles River, L'arbresle, France), weighing about 230–250 g, were acclimated to our animal house under controlled temperature (22 C) and light (12-h light, 12-h dark) conditions, with free access to water and to a control starch-enriched diet (SED). The composition of the SED was as follows: starch glucose (50%), protein (23,5%), lipids (4%), water (12%), mineral salts (5.5%), cellulose (4%), vitamins (1%), weight basis. The composition of the PED (isocaloric regarding the SED) was essentially the same with a modification of the starch/glucose to protein ratio, *i.e.* starch/glucose (23.5%) and protein (50%). Protein used was a mixture of soya protein and casein (50/50). Fasted rats (n = 5 per group) always had free access to water. PED-fed rats (n = 8) were switched on the PED for 3 d before studies. All experiments in fed rats were performed in the postabsorptive state, *i.e.* 6 h after food removal.

All protocols used in this work were performed according to the recommendations of our local animal ethics committee for animal experimentation.

Tissue sampling, metabolites, and gene expression analyses

Rats were anesthetized using an injection of pentobarbital (7 mg/100 g body weight). A laparotomy was performed to expose the liver and one kidney, which were rapidly removed and frozen using tongs previously chilled in liquid N_2. The frozen tissues were kept at −80 C until use. They were powdered at the temperature of liquid N_2 and homogenized by ultrasonication in 10 mmol/liter HEPES, 0.25 mol/liter sucrose (pH 7.3) (9 vol/g tissue).

Glycogen was determined according to the method described by Bergmeyer and Gawehn (17). Glc6Pase activity was assayed at maximal velocity (20 mmol/liter glucose-6 phosphate) at 30 C, by complex formation of the phosphate produced. The contribution of nonspecific phosphatase activities was estimated through hydrolysis of β-glycerophosphate, as previously described (3). Cytosolic phosphoenolpyruvate (PEPCK) carboxykinase (PEPCK-c) activity was assayed under conditions of maximal velocity at 30 C in the 100,000 × *g* supernatant of tissue homogenates, using the decarboxylation assay described by Rajas *et al.* (4) and Jomain-Baum and Schramm (18). Glutaminase activity (π-dependent) was assayed in kidney homogenates under conditions of maximal velocity, according to previously described procedures (6, 19).

For Western blotting studies, 30 μg protein was separated by electrophoresis in 9% polyacrylamide gels in the presence of sodium dodecyl sulfate. After electrotransfer to polyvinylidene fluoride immobilon membranes (Millipore Corp., Billerica, MA), immunodetection was performed using anti-Glc6Pase and anti-PEPCK (kindly provided by Dr. Granner, Valderbilt University Medical School, Nashville, TN) antibodies at dilutions of 1:1500 and 1:7000, respectively (4, 20). Membranes were reblotted with anti-β-tubulin (use at 1:2000; Santa Cruz Biotechnology, Inc., Santa Cruz, CA,) for standardization.

Total RNA was extracted following the protocol described by Sambrook *et al.* (21). cDNA synthesis was performed from 500 ng mRNA using Moloney murine leukemia virus Reverse Transcriptase ribonuclease H Minus (Promega Corp., Charbonnières, France) and poly(deoxythymidine) oligonucleotides, incubated for 1 h at 40 C. Real-time PCR was performed using a LightCycler (Roche Diagnostics, Mannheim, Germany). A standard curve was systematically generated with different amounts of a purified target cDNA fragment. Ribosomal protein rL19 transcript was used as a reference, and the results were expressed as a ratio referred to the expression of rL19. PCR was performed with a 1:100 dilution of the reverse transcription product in the Master SYBR Green I mixture (Roche Diagnostics) with specific primers (0.25 μM final of each primer). The following specific primers used were: 5′–TTACGAAGACTCCCAGGACTG–3′ (sense) and 5′–TGACTATTACAGCAACAGCTC–3′ (antisense) for the rat Glc6Pase, 5′–AGTGCCTGTGGGAAAACCAA–3′ (sense) and 5′–CACCATCTTCACCAACGTGGCT–3′ (antisense) for the rat PEPCK, 5′–AGATTGACCGTCATATGTATCACA–3′ (sense) and 5′–TCTAAGACCAAGGAAGCACGAA–3′ (antisense) for the rat L19, and 5′–GCACATTTCCCTCACCAAGT–3′ (sense) and 5′–TCTTTCCTGGTCCATCAACC–3′ (antisense) for the rat G6PC3.

Determination of kidney glucose fluxes

Anesthetized rats were fitted with polyethylene catheters inserted into the right jugular vein for 3[^{3}H]glucose infusion and into the left carotid artery for blood sampling, as previously described (5, 9). The body temperature was maintained at 37.5 C by a rectal probe-monitored heating blanket. Rats were infused for 90 min. In preliminary experiments we checked that a steady state of glucose-specific activity (SA) was obtained in the venous and arterial blood from 40–50 min and afterwards under our conditions of infusion. At the end of the infusion time, blood was sampled from the carotid artery and one renal vein simultaneously, as previously described (5, 9). Blood plasma was separated and used for determinations of glucose concentration and SA. Total EGP was calculated from the 3[^{3}H]glucose infusion rate and glucose SA (16). The fractional extraction (FX) of glucose was calculated as: (3[^{3}H]glucose SA $_{artery}$ × glucose concentration $_{artery}$) − (3[^{3}H]glucose SA $_{vein}$ × glucose concentration $_{vein}$)/(3[^{3}H]glucose SA $_{artery}$ × glucose con-

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centration $_{artery}$). The renal glucose uptake (RGU) was deduced from this FX, the arterial plasma glucose and the renal blood flow (RBF), as: RGU = RBF × plasma glucose concentration $_{artery}$ × FX. The determination of RBF using a radiolabeled microsphere technique was performed as previously described, from separate groups of rats (5). The RBFs determined were: 11.2 ± 0.8, 9.4 ± 0.6, 7.5 ± 1.0, 8.19 ± 0.5, 11.3 ± 1.0 m/min in SED-fed, and 24-h, 48-h, 72-h fasted and PED-fed rats, respectively. The renal glucose balance (RGB) was calculated from the difference between arterial and renal glucose concentration and RBF, as: RGB = RBF (glucose concentration $_{artery}$ − glucose concentration $_{vein}$). Finally, the renal glucose release (RGR) was deduced from RGB and RGU, using the equality RGB = RGU − RGR.

Determination of insulin effect on EGP and glucose utilization

PED and SED-fed rats were anesthetized using pentobarbital. Rats were equipped with catheters as described previously, in the left carotid artery for blood sampling and in the right jugular vein for infusion of 3[^{3}H]glucose, of insulin at either 240 or 2880 pmol/h, and of glucose (1.67 mmol/liter) at an adapted rate to maintain euglycemia (8, 16, 20). Glycemia was monitored every 15 min with a glucometer (OneTouch Ultra; LifeScan, Inc., Milpitas, CA) to adapt the infusion of glucose if necessary. After 90 min infusion, a laparotomy was performed to remove a freeze-clamp liver lobe. A blood sample was taken for plasma insulin and glucose SA determinations. Plasma insulin was assayed using a RIA procedure with an ultrasensitive rat insulin ELISA kit (Crystal Chem Inc., Downers Grove, IL). The glucose appearance and disappearance rates were calculated from the 3[^{3}H]glucose SA data (8, 16, 20). EGP was calculated from the appearance rate by subtracting the exogenous glucose infusion rate.

Statistical analyses

Data are presented as means ± SEM, and statistical analyses were performed using the Student's *t* tests (for paired or unpaired values as indicated). When appropriate, ANOVA tests were performed beforehand.

Results

Protein feeding stimulates gluconeogenesis gene expression in the kidney, but not in the liver

The Glc6Pase was increased about 2-fold in the kidney of PED-fed rats (n = 5), compared with SED-fed rats (n = 5). This took place at the level of mRNA, protein, and enzymatic activity assayed at the maximal velocity (Fig. 1, A–C). The induction of the PEPCK gene expression was even more marked. The PEPCK-c mRNA abundance was augmented by about 6-fold in the kidney of PED-fed rats compared with SED-fed rats (Fig. 1D). Similar increases at the protein and enzymatic activity levels were also observed (Fig. 1, E and F). Glutaminase activity was increased by 50% at the end point of maximal velocity (data not shown). A ubiquitously expressed nongluconeogenic Glc6Pase-related protein (called UGRP or G6PC3) is expressed in the kidney. No change was found in the kidney mRNA content of this protein in PED-fed rats compared with their SED-fed counterparts [2.9 ± 0.6 *vs.* 2.7 ± 0.5 Arbitrary Units (normalized with rl19 mRNA abundance), means ± SEM, n = 5], in agreement with previous results from others (22).

Unlike the results obtained in the kidney, no increase was noted either for the Glc6Pase or PEPCK in the liver of rats fed with the PED (Fig. 2). In keeping with the results at a protein level, no change was observed at a mRNA level (data not shown). No change was observed in the hepatic triglyceride content in PED-fed rats compared with SED-fed rats (7.6 ± 0.7 *vs.* 6.2 ± 0.6 mg/g liver, respectively, means ± SEM, n = 5).

Protein feeding markedly augments kidney glucose release

Next, we raised the question of the effect of PED-induced gluconeogenesis gene expression on glucose fluxes in the kidney. We used a combination of glucose tracer dilution analysis and arteriovenous glucose balance determination to separate the RGU and RGR, as previously used to quantify intestinal glucose fluxes (5, 9).

In SED-fed rats (n = 5), the 3[^{3}H]glucose SA was lower ($P <$ 0.05) in the renal vein than in the artery (∼−3.5%), indicating that unlabeled glucose was released by the kidney (Table 1). However, the plasma glucose concentration was not different in the vein compared with the artery, suggesting that both kidney glucose release and uptake were comparable. Accordingly, from the FX and RBF (11.2 ± 0.7 ml/min, mean ± SEM, n = 4), the RGR derived from the calculated RGB and RGU amounted to 12.5 ± 1.9 μmol/kg^{-1} · min^{-1} (Table 1). This represented about 17% of total EGP.

Because a quantification of kidney glucose release has never been performed using this approach in fed and fasted rats, we characterized kidney glucose production along fasting. In 24-h fasted rats (n = 5), the decrease in glucose SA in the renal vein was greater ($P < 0.05$) than that in 6-h postabsorptive rats (−9.5%) (Table 1). As in fed rats, there was no difference in plasma glucose concentrations in the renal vein and the artery. However, the FX was higher ($P < 0.05$) than that in fed rats (Table 1). In line with this FX increase, the RGU and RGB calculated from these data and from the RBF (9.8 ± 1.5 ml/min), RGR was markedly higher ($P < 0.05$) in 24-h fasted rats than in SED-fed rats, *i.e.* 22.3 ± 2.1 μmol/kg^{-1} · min^{-1}. This represented about 50% of EGP, the latter being lower in fasted rats than fed rats, in agreement with previous data (5, 20). Similar results were obtained from rats fasted for a longer time, *i.e.* 48 and 72 h (Table 1). In agreement with the rapid increase in kidney glucose release in the fasting situation, we observed a marked increase (by about six times) of the renal PEPCK amount from 24 h fasting (data not shown). Moreover, we previously reported the induction of the renal Glc6Pase gene in the fasted state (23).

In PED-fed rats (n = 8), there was a decrease (∼−6.5%) in glucose SA between the artery and the renal vein ($P < 0.05$) (Table 1). Arterial plasma glucose concentration was higher (+6%; $P < 0.05$) than the venous glucose concentration. The FX was close to zero and not different from that in SED-fed rats (Table 1). The RGR, derived from RGB and RGU, was equal to 28.2 ± 4.5 μmol/kg^{-1} · min^{-1}, *i.e.* 2-fold higher than that found in SED-fed rats ($P < 0.05$). This represented about 45% of total EGP, the latter being not significantly different than that in SED-fed rats (Table 1), in agreement with previous results (9).

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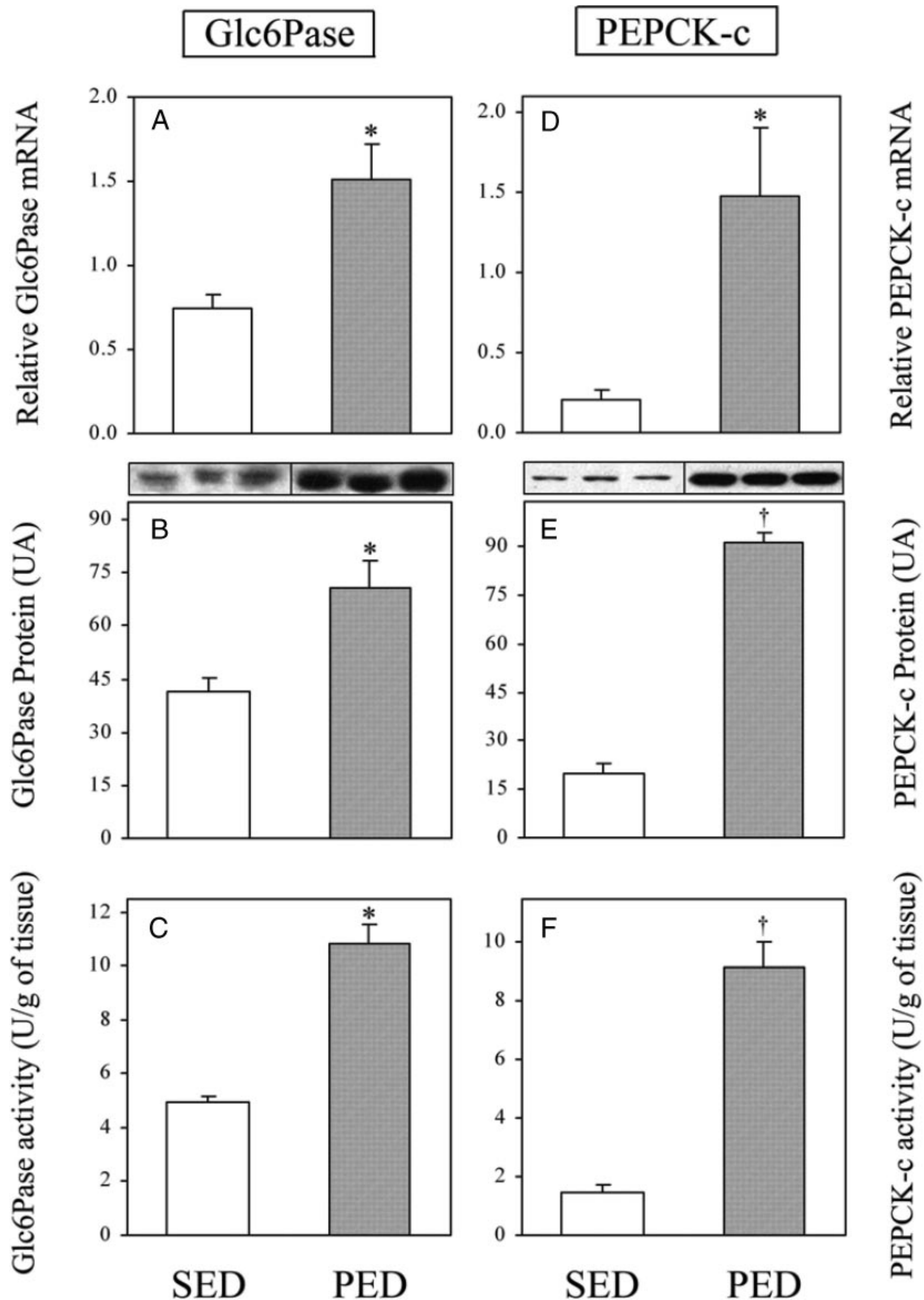

FIG. 1. Analysis of gluconeogenesis gene expression in the kidney of PED and SED-fed rats. A and D, Glc6Pase mRNA and PEPCK-c mRNA abundance, respectively, normalized with rl19 mRNA abundance (n = 4). B and E, Densitometric Western blot analyses of Glc6Pase and PEPCK-c, respectively, obtained from five animals under each condition (*upper panels*, representative analysis from three animals in each group). C and F, Glc6Pase and PEPCK-c activity, respectively, assayed under maximal velocity condition (n = 5). Data are expressed as mean ± SEM. *, †, Statistically different from SED value ($P < 0.05$ and $P < 0.01$, respectively, Student's *t* test for unpaired values).

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Effect of insulin infusion on EGP and glucose utilization

We addressed the question of insulin action on both EGP and glucose utilization in rats fed the PED compared with those on the SED. A low dose of insulin (240 pmol/h) was previously shown to partially inhibit EGP and a higher dose (2880 pmol/h) to obtain a maximal velocity of glucose utilization (16, 20, 24). There was no difference in basal EGP (saline infusion) in both groups (Fig. 3). EGP was suppressed by 25–30% by insulin in-

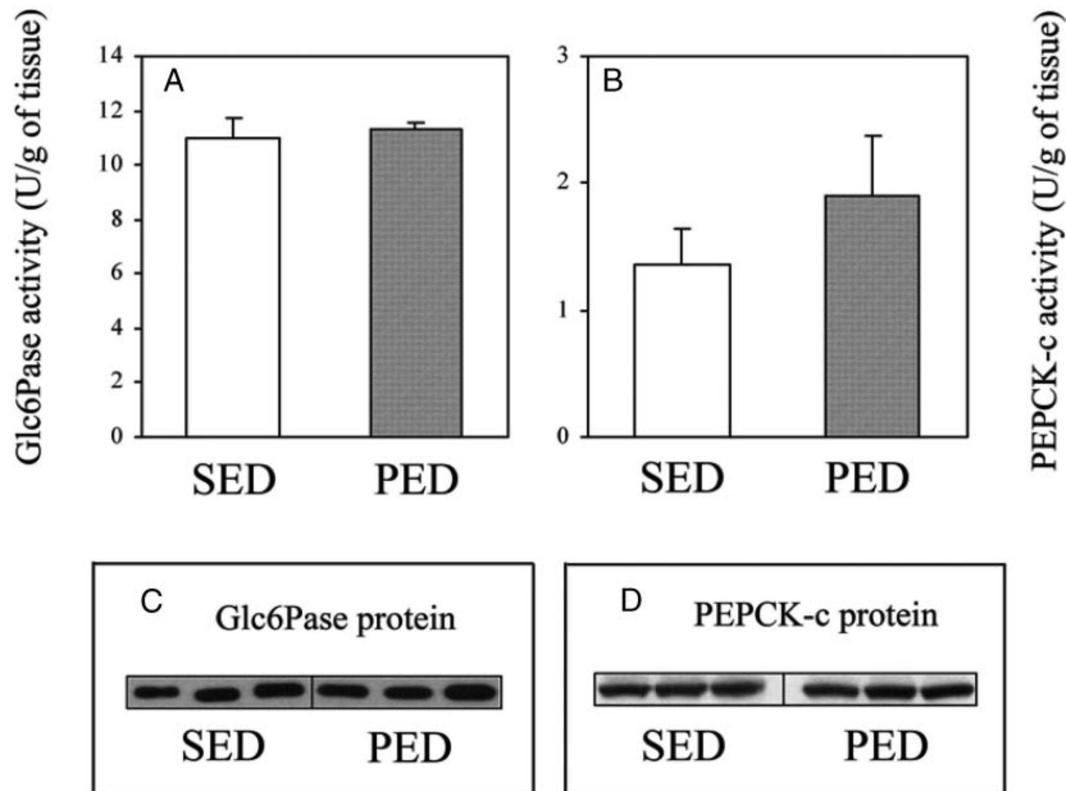

FIG. 2. Analysis of gluconeogenic enzyme activities in the liver of PED and SED-fed rats. A and B, Glc6Pase and PEPCK-c activity, assayed under conditions of maximal enzyme velocity (n = 5). Values are means ± SEM. C and D, Western blot analyses of Glc6Pase protein and PEPCK-c protein, respectively. Values are means ± SEM.

fusion (low dose) in rats fed the SED. The suppression was substantially stronger (~50%) in their counterparts fed the PED ($P < 0.05$ with regards to SED-fed rats). EGP was similarly suppressed (by at least 90%) in both groups of rats at the high-insulin dose (Fig. 3). There was no difference in glucose utilization at either dose of insulin infused. Plasma insulin levels at the end of clamp experiments were comparable in SED and PED-fed rats in either basal or insulin-infused conditions (Table 2).

Glycogen stores were studied in the liver of SED and PED-fed rats infused with 240 pmol/h at the end of the clamp experiment. The hepatic glycogen content was markedly higher in PED than SED-fed rats (18 ± 3.5 *vs.* 4.7 ± 3.2 mg/g wet liver; $P < 0.05$) (Fig. 4). Glycogen was not detectable in the kidney of either groups of rats.

Discussion

The dysregulation of EGP is known to be a crucial alteration of glucose homeostasis, likely involved in the development of type 2 diabetes. However, the mechanisms regulating the release of glucose from the organs responsible for EGP are still poorly characterized, especially regarding their control in various nutritional states (6, 25). A recent study has reported that PEDs

TABLE 1. Renal glucose fluxes in SED-fed, fasted, and PED-fed rats

Diet/fast	3[^{3}H]glucose SA (dpm/μmol)		Glucose concentration (mmol/liter)			μmol · kg^{-1} · min^{-1}			
	Artery	Vein	Artery	Vein	FX	RGB	RGU	RGR	EGP
SED	39,647 ± 2,467	38,244 ± 2,330[a]	8.6 ± 0.4	8.7 ± 0.4	0.02 ± 0.01	−6.3 ± 4.6	6.2 ± 3.2	12.5 ± 1.9	75.0 ± 4.9
Fasted									
24 h	39,748 ± 1,332	35,975 ± 1,296[a]	5.5 ± 0.1	5.5 ± 0.1	0.09 ± 0.02[b]	−1.8 ± 5.5	20.5 ± 5.4	22.3 ± 2.1[b]	40.2 ± 1.4[c]
48 h	43,080 ± 3,280	39,036 ± 3,008[a]	6.0 ± 0.1	6.0 ± 0.1	0.08 ± 0.03[b]	−1.8 ± 6.1	18.0 ± 5.9	19.8 ± 1.4[b]	39.1 ± 1.6[c]
72 h	40,673 ± 2,107	37,294 ± 2,093[a]	7.3 ± 0.2	7.6 ± 0.2[a]	0.04 ± 0.01	−13.2 ± 2.0	11.6 ± 2.5	24.8 ± 1.4[c]	45.0 ± 2.3[c]
PED	32,526 ± 873	30,255 ± 931[a]	9.0 ± 0.3	9.6 ± 0.2[a]	0.00 ± 0.02	−26.1 ± 6.3	2.1 ± 6.4	28.2 ± 4.5[b]	63.3 ± 1.1

The results are expressed as means ± SEM (n = 5 for SED-fed and fasted rats; n = 8 for PED-fed rats).

[a] Different from value in artery ($P < 0.01$, Student's two-tailed *t* test for paired values).

[b] Different from value in SED-fed rats ($P < 0.05$, ANOVA test, followed by Student's *t* test for unpaired values).

[c] Different from value in SED-fed rats ($P < 0.01$, ANOVA test, followed by Student's *t* test for unpaired values).

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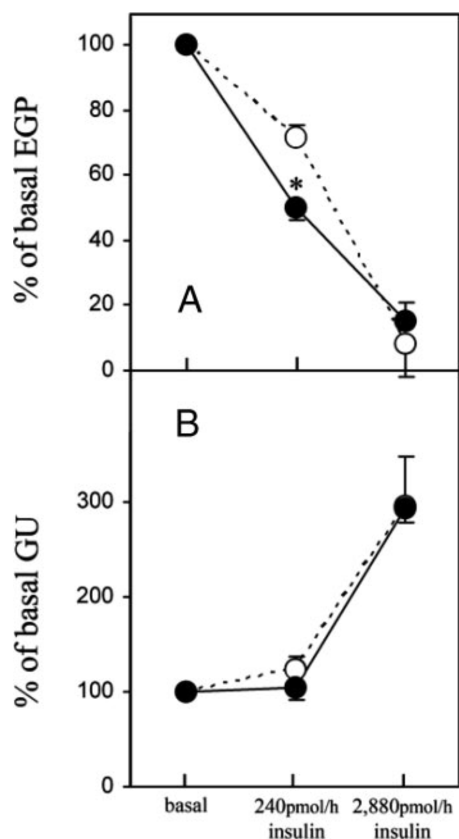

FIG. 3. Effect of insulin on glucose fluxes in SED (*dotted line*) and PED-fed (*full line*) rats maintained in euglycemia. A and B, EGP and glucose uptake (GU) were determined as described in the text. Basal EGP and glucose uptake were 75 ± 2 μmol/kg^{-1} · min^{-1} and 80 ± 5 μmol/kg^{-1}/ · min^{-1}, in SED and PED-fed rats, respectively. Values are means ± SEM. *, Statistically different from SED value ($P < 0.05$, Student's *t* test for unpaired values).

increase intestinal glucose release, without global variation of total glucose production. This has suggested a compensatory decrease of the contribution to EGP of at least one of the other gluconeogenic organs (*i.e.* the liver or kidney) (9). To study further this hypothesis, we characterized in this work the contribution of both the liver and kidney to glucose production under the same diet. To this aim we used both gene expression studies, and a combination of glucose tracer dilution and arteriovenous glucose balance determinations to quantify the organ glucose release.

We report herein that the rat kidney might contribute to a significant part of EGP (15–20%) in the postabsorptive state in rats fed a standard chow diet. Our results are in agreement with previous studies in fed postabsorptive dogs (26) and humans

TABLE 2. Plasma insulin concentration in SED-fed and PED-fed rats after a hyperinsulinemic euglycemic clamp

	Plasma insulin (pmol/liter)	
Insulin infusion rate (pmol/h)	**SED**	**PED**
0	204 ± 26	291 ± 54
240	895 ± 203	922 ± 66
2,880	16,907	17,463 ± 1,868

Values are the means ± SEM (n = 4).

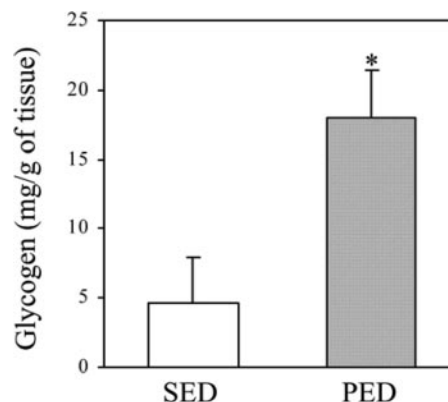

FIG. 4. Glycogen content in the liver of SED and PED-fed rats after a hyperinsulinemic euglycemic clamp performed with 240 pmol/h insulin (n = 4). Values are means ± SEM. *, Statistically different from SED value ($P < 0.05$, Student's *t* test for unpaired values).

(27). Interestingly, the kidney glucose release rapidly increases during fasting and might contribute about one half of EGP after 24 h. This sustained production then plateaus if fasting is prolonged. It must be noted that a similar contribution of the kidney to EGP was previously reported in fasted rats, based on either a liver removal approach (28), or when kidney vessels were ligated (29), and in long-term fasted humans (30).

It should be noted that despite evidence for kidney glucose release in both the postabsorptive and fasted states, there is no alteration of the renal net glucose balance (but in PED-fed rats), and, therefore, no net increase in glycemia in blood exiting the kidney. Thus, one may legitimately ask the question of the usefulness of this glucose release for the whole body. This relevant question has been raised by Malcolm Watford (15) in a comprehensive critical analysis of our previous works related to gluconeogenesis in the gut, which exhibits the same characteristics. Both the SI and kidney exhibit the other common feature to be high glucose-consuming organs (27–29). Without their gluconeogenic capacity, the blood plasma glucose concentration would certainly be strongly decreased by passage across these organs. This would be detrimental to other tissues of the body. Therefore, that both the kidney and SI are able, via their regulated gluconeogenic function, to counterbalance the lowering of glycemia resulting from glucose utilization, is of major usefulness for systemic glucose homeostasis, especially during fasting.

It is obvious that a critical data set in the approach we used relates to the estimation of glucose SA in the artery and vein. This has previously been pointed out by us (5, 8) and others (15, 27, 31). Because of interindividual differences among rats, which may be complicated by the relative inaccuracy of radioactivity determination and the high blood flow, there is a large dispersion in the mean SA data. This may cast doubts about what data are analyzed and how, when significance is established from such dispersed data sets, as pointed out (15). Regarding this particular point, venous and arterial glucose is obtained from the same animal. Thus, statistical analyses may be performed using paired test analyses (5, 6, 8, 9). This allows one to evidence differences between arterial and venous glucose SA, even if the means are very close and obtained from variable data (Table 1), because the sense of variation is tested and not the mean (9). An additional

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difficulty is that these small differences have to be multiplied or divided by other values (*e.g.* blood flow), which themselves are inaccurate, to be converted into glucose fluxes. Moreover, because the gold method microsphere technology necessarily introduces perturbations of the blood microcirculation, the blood flow cannot be determined in the rats infused with 3[^{3}H]glucose. Thus, RBFs were determined in separate groups of animals, and a single mean value was used for all animals within a group. The resulting calculated glucose fluxes, thus, display large variations (5, 8, 15). As a related point, FX values sometimes are not different from zero, which is theoretically impossible for glucose-consuming organs. This problem has been encountered by us herein (Table 1) and in previous studies (5), and by others, which are renowned experts in the field (27, 31). As a consequence, even when differences in arterial and venous glucose SA are significant (and so attest of a release of glucose), the final calculated glucose fluxes constitute estimations and not, from a quantitative point of view, definitive values. It should be noted that we determined very comparable values of kidney and gut glucose fluxes from a vessel-ligating approach, which allowed us to free of the difficulties linked to the high blood flow and to the determination of arterial and venous glucose SA (29). An interesting observation relates to the higher glucose removal (as revealed from FX) in fasted rats compared with SED-fed rats (Table 1). The fasting state is characterized by increased lipolysis and proteolysis, which are both associated with increased acidosis and ammoniagenesis. This may warrant a higher metabolic demand for the kidney in fasted states compared with the fed states.

It must be mentioned that a recent study questioned both *in vitro* and *in vivo* the occurrence of the release of glucose by the SI in 72-h fasted rats (32). It was concluded that glutamine gluconeogenesis is undetectable in the SI of 72-h fasted rats. The *in vitro* approach involved a SI ring mixture composed of samples taken along the whole SI length (including the distal ileum), with the aim to mimic the *in vivo* structure of the SI (32). We previously highlighted that the ileum exhibits at best a very weak expression of the gluconeogenic enzymes with regards to the duodenum and proximal jejunum (3, 5). This experimental choice could result in a high proportion of glucose-consuming cells, making it possible *in vitro* a reuse of glucose potentially produced. Despite this possible reuse, a glucose production, considered as "very small" by the authors, was detected after incubation in the presence of glutamine (by enzymatic determination) (32). This is in agreement with previous data from numerous *in vitro* studies reporting glucose production from either jejunal mucosa or perfused intestines obtained from rodents or humans (33–36). However, ^{13}C-glucose was not detected after incubation of the SI ring mixture with ^{13}C-glutamine, using a ^{13}C-nuclear magnetic resonance approach (32). Because of the necessity of a sophisticated treatment of the ^{13}C-nuclear magnetic resonance signal, to eliminate the background noise, it is known that a weakness of the approach is its low capacity to detect low concentrations of ^{13}C-metabolites. This may explain the discrepancy with the detection of glucose by the enzymatic method. In the *in vivo* study, the authors failed to evidence a net glucose release. They even observed a significant net glucose extraction, *i.e.* the portal vein glucose concentration was lowered compared with the arterial one. This was interpreted as a discrepancy with our net glucose balance data: we measured a net balance equal to zero, *i.e.* plasma glucose concentrations were the same in the portal vein and the artery in 72-h fasted rats (6). This slight discrepancy could be explained as follows. To optimize the determination of tracer dilution and, thus, of intestinal glucose release, we removed the blood flow irrigating the large intestine by ligaturing the related vessels (5, 6). The latter exhibits the capacity of glucose utilization, but not that of glucose production (3). Another choice was done in Ref. 32, *i.e.* to not remove this blood from the determinations. As a consequence, the capacity of glucose utilization of the gut system studied in Ref. 32 was higher than that we studied (6). This may warrant why a net glucose extraction by the SI was observed in Ref. 32, and not in Ref. 6. It is well known that the metabolic complexity of the SI makes it able to either use or produce glucose, sometimes simultaneously (for reviews, see Refs. 7 and 36). A similar reasoning may be applied to glutamine and various precursors (36). Therefore, reasoning about the utilization and release of glucose in the SI (a comparable rationale may be applied to the kidney) cannot be based solely on net glucose balance determinations. It definitively requires the separation of the actual uptake and the actual production of glucose, which is possible by combining net balance determination and tracer dilution (see herein and Refs. 5 and 9). This was not performed in the study by Martin *et al.* (32). Moreover, we previously showed that a net glucose uptake is quite compatible with the occurrence of a glucose release, *e.g.* in the SI of 48-h fasted rats (5).

Despite the limitations of our approach, we here report that kidney glucose release of PED-fed rats is increased and might account (with the cautions exposed previously) for about 45% of EGP in the postabsorptive state. Moreover, this results in a small net glucose release. In agreement with this result, the mRNA expression and the activity of renal gluconeogenic enzymes (PEPCK, Glc6Pase) are dramatically increased in PED-fed rats compared with SED-fed rats (results of Fig 1). In contrast, the protein activities of hepatic PEPCK and Glc6Pase measured at maximal velocity are not modified by the PED. We recently reported in rats that the same PED induces intestinal glucose production (IGP) by increasing the activities of gluconeogenic enzymes (9). In this previous work, we estimated that IGP might account for about 20% of EGP in PED-fed rats. Moreover, EGP was altered by PED in neither the previous nor the current study. Together, our data suggest that protein feeding induces redistribution of glucose fluxes among gluconeogenic organs. Because both RGP and IGP are induced without global increase of EGP, this suggests a blunting of liver glucose production. By combining RGP to IGP, one might infer that the production of glucose by the liver might represent less than 50% of EGP in PED-fed rats. This might appear unlikely because the liver is generally considered as the main glucose-producing organ of the body in the postabsorptive situation (1, 2). However, one must keep in mind that in another special situation, *e.g.* long-term fasting (72 h in the rat), the same inference could be made, as we previously suggested that the SI glucose release might possibly account for about one third of EGP (6, 7) (Table 1). Obviously, these assumptions should be regarded in light of the cautions about ac-

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curacy exposed previously. However, arguing for a minored role of the liver in long-term fasting, we previously reported that hepatic Glc6Pase gene decreases from 48 h fasting, which is associated with a marked rebound of their glycogen stores (37, 38). Interestingly, we observed in separate unpublished studies that the hepatic glycogen stores are higher ($P < 0.05$) in the liver of PED-fed rats than SED-fed rats (36 ± 3 *vs.* 26 ± 2 mg/g liver, means ± SEM, n = 4). Thus, the blunting of hepatic glucose release might occur and benefit of hepatic glycogen storage in PED-fed rats as in long-term fasting rats. It must be mentioned that a recent study dealt with the effect of protein feeding on gluconeogenic genes in the liver, kidney, and SI in the rat. The respective tissue mRNA contents of PEPCK, Glc6Pase, and G6PC3 [a ubiquitous Glc6Pase-related protein, known to be nongluconeogenic (39)] were studied. Interestingly, PEPCK and Glc6Pase mRNA contents were higher in the SI of high-protein fed rats than in the SI of low-protein fed rats, and the glycogen content was also higher in the liver of overnight-fasted high-protein fed rats (22). Both observations corroborate our own data (see herein and Ref. 9).

We then raised the question to know whether a PED may improve insulin sensitivity in rats. We chose to perform hyperinsulinemic euglycemic clamp. This is the gold method, compared with glucose and/or insulin tolerance tests, because it allows us to assess insulin sensitivity, separating the effect on either production or utilization of glucose. We demonstrate that protein feeding markedly ameliorates the suppression of EGP by insulin (results of Fig. 3 and Table 2), whereas having no effect on peripheral glucose uptake. Gluconeogenesis is considered less sensitive to inhibition by insulin than glycogenolysis. Thus, one may hypothesize that the improvement of insulin suppression of EGP in PED-fed rats might mainly concern the glycogenolysis pathway. In keeping with this proposal, hepatic glycogen stores are higher in PED-fed rats at the end of the clamp experiments compared with SED-fed rats (results of Fig. 4). The liver is a main contributor to glucose production in various situations and the sole among gluconeogenic tissues to be capable of glycogen storage. Therefore, it is likely that the improvement of insulin sensitivity of liver glycogenolysis should have major beneficial effects on glucose homeostasis, not only in rats but also in normal and/or insulin-resistant human subjects. As a related point, recent human studies show that protein feeding in type 2 diabetic patients markedly improves glucose tolerance (12–14). Conversely, impaired liver glycogen storage due to uncurbed hepatic glycogenolysis has been a key feature involved in the anomalies of regulation of EGP in type 2 diabetic patients (40).

Including our results herein, there now exists a body of arguments arguing for the beneficial effects of food protein in glucose homeostasis in obese and diabetic patients. We previously reported in the rat that protein diet induces glucose release by the intestine, initiating satiety signals to the brain (9). This is in line with the satiety effects induced by food protein in humans (11). Moreover, it is known that the presence of glucose and insulin in the portal blood is necessary and sufficient to suppress hepatic glucose release and promote glycogen storage (10, 20). This is in agreement with our results of a blunting of glucose production in the liver on the benefit of the two other gluconeogenic tissues. This is also in keeping with the improvement of the suppression of EGP and the promotion of hepatic glycogen storage by insulin reported herein. Finally, our results might explain at least in part the rapid and spectacular improvement of glucose homeostasis observed in type 2 diabetic patients, upon increasing the proportion of protein in their diet (12–14).

In conclusion, we have better defined here the mechanisms regulating EGP under the specific nutritional condition that represents a protein-rich diet. Our results highlight the key importance of a redistribution of glucose production among gluconeogenic organs in the improvement of glucose homeostasis. Thus, targeting specific sites of gluconeogenesis either by pharmaceutical or nutritional approaches might constitute an attractive approach to correct the defaults of glucose metabolism in type 2 diabetic patients.

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