Glucose-6-Phosphatase mRNA Levels in Kidney Isolated Tubule Suspensions Are Increased by Dexamethasone and Decreased by Insulin

Hélène Simonnet

The strong induction of renal glucose-6-phosphatase (G6Pase) during starvation has been suggested to be responsible for the increased role of the kidney in glucose production during long-term fasting. To investigate whether this induction may be caused by a direct hormonal effect on the renal proximal tubular cell, we incubated rat renal tubule suspensions in the presence of glucocorticoids or insulin for 6 hours; normoxia was required, since hypoxic conditions were associated with markedly decreased G6Pase mRNA levels despite maintenance of adenosine triphosphate (ATP) levels. The G6Pase mRNA level was increased twofold to threefold by 10^{-8} to 10^{-5} mol/L dexamethasone (DXM), whereas the most effective concentration of insulin, 10^{-9} mol/L, induced only a 40% decrease. These results suggest that the increased role of the kidney in glucose production during long-term starvation could be linked to a direct effect of glucocorticoids on renal G6Pase.

IT HAS BEEN SUGGESTED that the strong induction of renal glucose-6-phosphatase (G6Pase) during starvation is responsible for the increased role of the kidney in glucose production during long-term fasting.[1,2] This induction could be caused directly or indirectly by numerous factors, among which are increased plasma glucocorticoids[3,4] or decreased insulinemia.

Glucocorticoids have long been recognized as important modulators of glucose production and gluconeogenic enzymes, especially during starvation.[5,6] However, a direct effect of glucocorticoids on G6Pase expression has not been clearly established by in vivo studies, since numerous hormones (insulin or catecholamines) or metabolites (glucose) are produced in response to glucocorticoid treatment. A direct effect was suggested by the fact that glucocorticoid response elements were found in promoter regions of several genes encoding gluconeogenic enzymes such as G6Pase and phosphoenolpyruvate carboxykinase (PEPCK).[7,8] However, to date, no conclusive information has been obtained about the effectiveness of these consensus sites in any of the two major gluconeogenic cell types, ie, hepatocyte or renal proximal cell. This is due to the difficulty of studying gene regulation in freshly isolated cells, which remain viable for only 1 or 2 hours, and in cell lines that frequently lose or gain regulatory properties, rendering the results obtained with these models unsatisfactory. Indeed, in FAO hepatoma cells, dexamethasone (DXM) induces a biphasic increase of G6Pase mRNA, whereas in cultured hepatocytes no change in G6Pase mRNA is induced by DXM after 24 hours[7]; no data are available for renal cells.

In this study, we used suspensions of renal tubule fragments incubated for 6 hours with DXM or insulin to determine whether these compounds can alter the expression of G6Pase by a direct effect on the renal tubular cell. Moreover, since PEPCK, another gluconeogenic enzyme, was shown to be repressed by mild hypoxia,[9] we checked the effect of Po_2 on G6Pase mRNA levels and determined the optimal conditions for tubule oxygenation.

From the Laboratoire Mécanismes Moléculaires du Diabète, Institut National de la Santé et de la Recherche Médicale (INSERM U449), Faculté de Médecine Laënnec, Lyon, France.

Submitted November 20, 1998; accepted February 25, 1999.

Supported by INSERM.

Address reprint requests to Hélène Simonnet, MD, PhD, Centre de Génétique Moléculaire et Cellulaire, CNRS UMR 5534, Université Lyon 1, 43, Bd du 11 Novembre 1918, 69622 Villeurbanne Cédex, France.

0026-0495/99/4808-0021$10.00/0

MATERIALS AND METHODS

Materials

Dulbecco's modified Eagle's medium (DMEM)/Ham F12 medium was obtained from Life Technologies (Cergy-Pontoise, France). Egg-white lysozyme, insulin, and DXM were from Sigma (St Louis, MO). Bovine serum albumin, collagenase A, and DNase A were from Boehringer (Mannheim, Germany). The rat G6Pase cDNA probe was previously cloned by this laboratory.[10] The ^{32}P-CTP was obtained from ICN Biomedicals (Orsay, France), and the cDNA probe was labeled with a Hexaprimer kit from Appligene (France). The human 18S cDNA probe was a gift from Dr George Stepien (Centre de Génétique Moléculaire et Cellulaire, Lyon, France).

Animals

Renal tubules were obtained from male Sprague-Dawley rats (IFFA-CREDO, L'Arbresle, France) weighing 200 to 300 g and feeding on a standard laboratory diet (UAR, Epinay-sur-Orge, France). Fed animals were killed at 9AM by intraperitoneal injection of 3 mg sodium pentobarbital/100 g.

Incubation Medium

The medium for tubule preparation and incubation was a 50:50 mixture of DMEM/HAM F12 containing 10 mmol/L HEPES, 25 mmol/L sodium bicarbonate, 8 mmol/L glucose, 2 mmol/L glutamine, 0.5 mmol/L lactate, and 20 mg lysozyme/L to decrease bacterial contamination. After sterilization by filtration, the medium was equilibrated with 5% CO_2 and the pH was adjusted to 7.4 at 37°C.

Tubule Preparation

Tubules were prepared at 4°C by a modification of the collagenase method of Vinay et al.[11] Four kidneys were excised from two anesthetized rats into cold sterile medium equilibrated with 5% CO_2 and 20% O_2. Dissected cortical tissue was forced through a 1-mm nylon mesh, and the rinsed fragments were incubated for 45 minutes in 15 mL medium with 25 mg bovine serum albumin, 15 mg collagenase, and 50 μg DNase at 37°C in the presence of 5% CO_2 and 20% O_2. The incubation was stopped with cold medium, and the suspension was filtered through a steel strainer (0.5-mm mesh) and rinsed three times by suspension in cold sterile medium and centrifugation for 1 minute at 200 x *g*.

Incubation Procedure

Immediately after preparation, 2.5 mL tubule suspension (3.3 ± 1.4 mg protein/mL) was placed into cylindrical 25-mL stoppered flasks equilibrated with 5% CO_2 and 20% O_2 (standard conditions), and then constantly shaken (120 strokes/min) at 37°C for 6 hours. Hormone effects were investigated by the addition to the suspension of 25 μL insulin or DXM at various concentrations. At the end of the incubation period, 2-mL aliquots of the suspension were immediately pelleted at 4°C (3 minutes at 200 *g*) and stored at −80°C until analysis for mRNA or G6Pase activity. Adenosine triphosphate (ATP) was determined in separate flasks after protein precipitation in 2% $HClO_4$ and storage at −20°C.

Glucose Production

At the end of tubule preparation or after 5 hours of incubation, 2.5-mL aliquots of tubule suspension were washed three times and resuspended in 2.5 mL Krebs-Henseleit bicarbonate buffer containing 2 mmol/L glutamine as the sole substrate, incubated for 1 hour in stoppered flasks with 5% CO_2 and 20% O_2, and shaken as before at 37°C. After sampling 100 μL for protein measurement, the contents of the flask were deproteinized with 2% $HClO_4$ and stored at −20°C for glucose determination.

Physical and Biochemical Determinations

Proteins were determined with the Bio-Rad (Issy-sur-Seine, France) procedure. ATP and glucose were assayed after neutralization by spectrophotometric methods.[12,13] The partial pressure of oxygen (Po_2), Pco_2, and pH of the incubation medium were determined with a blood gas analyzer. G6Pase activity was measured after thawing and solubilization of pelleted samples in 0.5% sodium cholate at saturating substrate concentration (20 mmol/L) using the method of Minassian and Mithieux.[1] The nonspecific phosphatase activity was measured with 20 mmol/L β-glycerophosphate as substrate, and subtracted from the total activity.

G6Pase mRNA Determination

After thawing of pelleted samples in 1.5 mL 4-mol/L guanidium thiocyanate sarcosyl solution, total RNA was extracted by the phenol/chloroform method of Chirgwin et al.[14] RNA was quantified by absorbance at 260 nm ($OD_{260}/OD_{280} > 1.8$), and the absence of degradation was checked by electrophoresis of a 1-μL aliquot of RNA. The relative abundance of G6Pase mRNA was determined by Northern blot hybridization analysis of 8 to 12 μg total RNA, as described by Mithieux et al.[10] G6Pase mRNA radioactive spots were quantified with a Bio-Rad Phosphorimager; total mRNA fixed onto the membrane was evaluated by hybridization to an 18S cDNA probe, and the results were expressed as the ratio of G6Pase mRNA radioactivity to 18S mRNA radioactivity.

Calculations

The results are expressed as the mean ± SEM and were analyzed using Student's *t* test.

RESULTS

Control of Tubule Viability and Function After 6 Hours of Incubation

Most previous studies involving tubule incubations were performed in a 95% O_2 atmosphere to ensure an unlimited O_2 supply to cells; however, oxygen cytotoxicity after 2 or 3 hours[15] prevents the use of tubule suspensions beyond this time. Therefore, we first defined a protocol for incubation and shaking in a physiological, nontoxic, 20% O_2 atmosphere, to maintain both viability and oxygen supply for 6 hours and to observe changes in mRNA levels.

Under the standard incubation conditions using 2.5 mL suspension per flask, the pH was 7.41 ± 0.06 (n = 4) and normoxia was maintained (Table 1). The total tubular ATP level at the end of the 6-hour incubation period was 25.6 ± 4.7 nmol · mg protein^{-1} (n = 7). This slightly elevated value is similar to the level obtained with a comparable model[16] and with cultured cells,[17] and is representative of metabolically active cells.

The gluconeogenic capacity of the tubular suspension was maintained after 5 hours of incubation, since tubules in Krebs-Henseleit medium in the presence of 2 mmol/L glutamine produced 53 ± 14 nmol glucose · h^{-1} · mg protein^{-1} (n = 4), similar to the value measured immediately after tubule preparation (50 ± 11 nmol glucose · h^{-1} · mg protein^{-1}, n = 4). It cannot be excluded that the former value is an overestimation because of the elimination of cellular debris during the washing procedure; the decrease of G6Pase activity after 6 hours (0.12 ± 0.03 μmol · min^{-1} · mg protein^{-1} *v* 0.17 ± 0.02 μmol · min^{-1} · mg protein^{-1} immediately after preparation) is perhaps a better estimation of the loss of gluconeogenic function. The G6Pase mRNA level of the fresh tubule preparation was, at best, half that of a freeze-clamped tissue. It increased to 150% of the time 0 value after 1 or 2 hours of incubation, probably reflecting the restoration of cell function, and then decreased to 85% of the time 0 value at 6 hours, paralleling the decrease in G6Pase activity.

Taken as a whole, these results demonstrate that no major alteration of cellular viability or function occurred during the course of the incubation.

Oxygen Requirement for G6Pase Expression at the mRNA Level

Increasing the volume of the incubation mixture in the flask (without changing its composition) greatly modified the oxygenation status, since hypoxia occurred in flasks containing an assay volume of 5 mL. Table 1 shows that under this condition, the G6Pase mRNA level decreased despite a constant ATP level.

Effect of DXM and Insulin on G6Pase mRNA Levels

Exposure of renal tubule suspensions to DXM for 6 hours induced a severalfold increase of G6Pase mRNA for each concentration tested (Fig 1). Exposure to insulin for the same time produced an opposite effect, but the decrease remained

Table 1. Relationship Between Po_2 and G6Pase mRNA Level

Parameter	Incubation Volume	
	2.5 mL	5 mL
Relative abundance of G6Pase mRNA (% of control)	100 (3)	53 ± 10 (3)
Po_2 (mm Hg)	117 ± 24 (4)	33 ± 10 (4)
Pco_2 (mm Hg)	43 ± 5 (4)	64 ± 3 (4)
ATP (nmol · mg protein)	26 ± 2 (7)	23 ± 1 (11)

NOTE. Values are the mean ± SEM of separate experiments (number in parentheses). Po_2 and Pco_2 of incubation medium were determined at the midpoint of the incubation (3 hours) and the G6Pase mRNA level and cellular ATP content at the end of the incubation (6 hours).

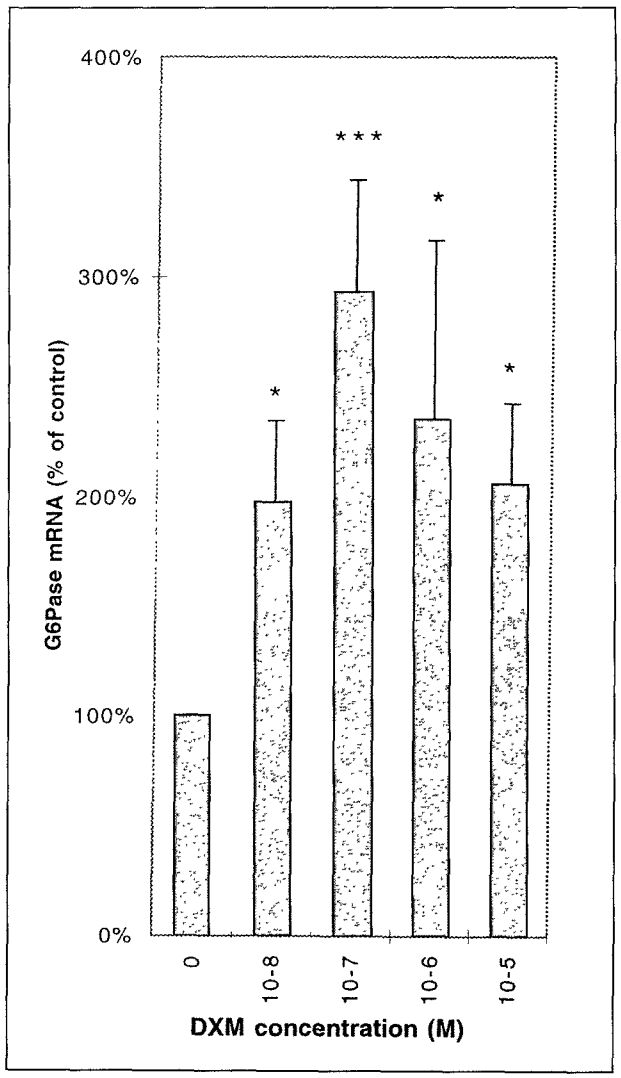

Fig 1. Effect of DXM on G6Pase mRNA levels. Renal proximal tubules were incubated for 6 hours in the presence of various concentrations of DXM, and the relative abundance of G6Pase mRNA was determined at the end of the incubation. Results are expressed as a percent of the value in the absence of hormone (mean ± SEM of 3 separate preparations). *$P < .05$, ***$P < .001$.

moderate (about 40%). Maximal inhibition was observed with 10^{-9} and 10^{-7} mol/L (Fig 2); higher concentrations had no additive effect (data not shown).

No significant change in G6Pase activity (not shown) was observed at any of the hormone concentrations tested, suggesting that 6 hours was not a sufficient amount of time to induce a change at the protein level.

DISCUSSION

Renal proximal tubular cells contain all of the enzymes required for glucose production and can produce glucose in vitro and in vivo.[18-20] During the fed state, or the early postabsorptive state, net renal glucose production is not measurable, since renal glucose utilization by the distal tubules and medulla compensates for the proximal tubule glucose production (28% of overall glucose turnover).[19,21] Inhibition of gluconeogenesis by anesthetics has been proposed to explain the conflicting results concerning renal net glucose output during short-term starvation.[22-24]

By contrast, the important role of the kidney in glucose homeostasis during prolonged starvation is widely accepted[18,20,25]; in this situation, the role of the kidney increases proportionally compared with that of the liver.[25] Actually, G6Pase has been shown by this laboratory to be increased in the kidney during long-term starvation, whereas the hepatic enzyme is no longer increased.[1,2] Other gluconeogenic enzymes are not differentially induced in the liver and kidney,[26] making G6Pase a good candidate to explain the increased role of the kidney in this situation,[2,10] possibly by means of a different hormonal sensitivity.

This study shows that both glucocorticoids and insulin are able to regulate the G6Pase mRNA level in the kidney proximal tubular cell.

Insulin is known to produce slight inhibition of renal gluconeogenesis in vivo,[19] in renal tubule suspensions incubated for 3 hours,[27] and in kidney slices after 1 hour of incubation.[28] In addition, the elevated level of renal G6Pase in insulinopenic streptozotocin-treated rats was reportedly normalized by insulin, suggesting that insulin was able to decrease expression of the G6Pase gene at the mRNA level after 12 hours and at the protein level after 24 hours.[10] The present study demonstrates that insulin is able to decrease renal G6Pase

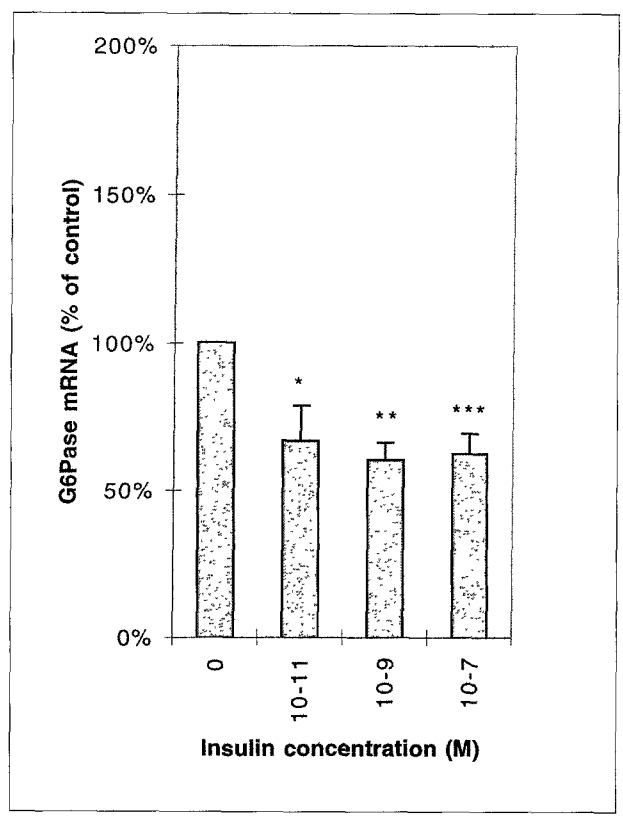

Fig 2. Effect of insulin on G6Pase mRNA levels. The protocol was similar to that for Fig 1, except that insulin replaced DXM. Results are from 4 separate preparations (mean ± SEM). *$P < .05$, **$P < .001$, ***$P < .0001$.

mRNA by a direct effect on the tubular cell. The decrease (−40% after 6 hours) is not inconsistent with the previously observed indirect effect in vivo (−70% after 12 hours[10]): it probably would have been much greater if the tubule preparation were obtained from insulinopenic rats, in which G6Pase is increased. However, it is also conceivable that in vivo, additional factors could contribute to the greater reduction of G6Pase mRNA levels.

This study shows that after 6 hours, DXM induced a much greater alteration of G6Pase mRNA levels than insulin in a renal in vitro model. In vivo, Joseph and Subrahmanyam[6] have shown that cortisone treatment for 5 days increases the activity of all gluconeogenic enzymes in the kidney, including G6Pase. The in vivo glucocorticoid-induced increase of G6Pase activity was confirmed by Voice et al[29] in liver and kidney.

These results and numerous other studies argue in favor of a different hormonal sensitivity between the liver and the kidney. Indeed, glucose production and G6Pase mRNA have been shown to be more sensitive to insulin or refeeding in the liver compared with the kidney,[7,10,18,30] making insulin a relatively specific modulator of liver glucose release. Conversely, in vivo, administered glucocorticoids, which are able to increase G6Pase activity in the kidney,[6] induce a smaller increase or no increment of G6Pase maximal activity in the liver.[29,31] Similarly, in vitro experiments showed no effect of DXM on G6Pase mRNA in cultured hepatocytes,[7] whereas the present study shows a marked increase in G6Pase mRNA following exposure of kidney tubules to DXM. It should be stressed that G6Pase is possibly part of a membrane complex in the reticulum, with the G6Pase active site inside the lumen associated with transporters for G6P[32] and for the products of the reaction, and possibly with other proteins[31] coded by different genes. The results presented here represent expression of the G6Pase gene, mRNA, and protein, as attested by enzymatic measurements on detergent-treated extracts.

Taken as a whole, these observations support the view that the increased glucose production by the kidney during prolonged starvation could be explained by increased glucocorticoids, since these hormones have been shown to increase gradually during fasting, especially in the Sprague-Dawley rat,[4] paralleling the increase of G6Pase,[1] whereas the decrease of plasma insulin is an early event of fasting.

A physiological 20% O_2 atmosphere and appropriate incubation conditions allowed the maintenance of normal Po_2 and viable tubule suspensions for up to 6 hours and thus the observation of G6Pase mRNA regulation. This amount of time appeared insufficient to observe modifications at the protein level; however, it seems to be the limit for shaken suspensions because of the mechanical stress to cells. The use of other biological models, renal proximal tubular cells in primary culture or renal cell lines,[17,33] has been excluded since they lose their oxidative gluconeogenic phenotype, and particularly G6Pase activity,[34] possibly in relation to the lack of oxygen in culture.[35,36] Therefore, improving the currently available models would be beneficial for the in vitro long-term study of G6Pase regulation.

The link between Po_2 and the G6Pase mRNA level in kidney cells is of interest, since it is apparently a direct effect of hypoxia, not associated with a decreased ATP level. Another gluconeogenic enzyme, PEPCK, has been shown to be inhibited by hypoxia in the liver.[9] Interestingly, in the kidney/liver-specific erythropoietin gene, which is strongly regulated by hypoxia, HIF-1 (hypoxia-inducible factor) binds DNA at a site close to the binding site for HNF-4, a transcription factor specific for gluconeogenic tissues,[8] and the two sites interact.[9] In vivo, the oxygen supply to kidney proximal tubular cells may be reduced transiently in strenuous exercise[37] or progressively in pathological obstruction of glomerular capillaries.[38] Therefore, it would be interesting to know if the hypoxia sensitivity of gluconeogenic genes is physiologically relevant.

ACKNOWLEDGMENT

Thanks are due to Annick Depin for her collaboration and to Carine Zitoun for excellent technical advice and kindness. I am also indebted to Professor Tom Riou and Dr Catherine Godinot for helpful discussions.

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