Stimulation by Insulin of Glucokinase Gene Transcription in Liver of Diabetic Rats*

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The purpose of this work was to investigate the molecular mechanism responsible for the induction of hepatic glucokinase in diabetic rats acutely treated with insulin. Experimental diabetes was provoked by injection of streptozotocin 8–10 days before the experiments. Regular insulin was given by three intraperitoneal injections at 8-h intervals, and the time course of glucokinase induction was followed over a time period of 24 h. The amount of glucokinase in liver was estimated by Western blotting of total cytosol protein with affinity-purified antibodies, as well as by conventional enzyme activity assays. Both measurements showed that glucokinase was reduced by more than 90% in the livers of diabetic rats as compared to normal controls. Following insulin administration, the amount (and activity) of glucokinase increased in a time-dependent fashion, after an initial lag of 4 h, to reach 65% of the nondiabetic control level 24 h after the initial dose of insulin. Northern blot analysis with a cloned cDNA probe was used to quantitate glucokinase mRNA. In contrast with the slow onset of enzyme accumulation, the amount of glucokinase mRNA was shown to be increased dramatically as early as 1 h after insulin administration. The abundance of specific mRNA increased until 8 h after the initial dose of insulin. Subsequently, the level of the mRNA decayed rapidly so that little message was left after 16 h and virtually none after 24 h. Run-on transcription experiments with isolated nuclei showed that the rate of transcription of the glucokinase gene was increased about 20-fold within 45 min of insulin administration and returned to the prestimulation level after 8 h. From these data, it was concluded that the induction of glucokinase resulted primarily from a burst in the transcriptional activity of the gene, leading to a short-term accumulation of glucokinase mRNA. The more sustained elevation of the enzyme level could be accounted for by the long half-life of the enzyme (>30 h). The virtually immediate activation of glucokinase gene transcription suggests a direct effect of insulin on the liver cell.

The liver-specific hexokinase isoenzyme (ATP::D-hexose 6-phosphotransferase, EC 2.7.1.1.), termed hexokinase type IV or hexokinase D and commonly referred to as glucokinase, is thought to play a key regulatory role in hepatic glucose metabolism. First, its high Kₘ for glucose allows for immediate adjustments in the rate of glucose phosphorylation in function of the plasma glucose concentration (1). Second, its specific activity is subject to long-term changes depending on the nutritional and hormonal status of the animal (2). Glucokinase activity in liver is low during fasting and in diabetes mellitus. It increases after carbohydrate ingestion or in diabetic animals after insulin treatment (3, 4). Physiologically, a low glucokinase activity favors the release into the circulation of glucose synthesized via gluconeogenesis. Conversely, a high glucokinase activity promotes glycogen deposition in the liver (5). The level of hepatic glucokinase activity appears to be determined essentially by regulation of the rate of enzyme synthesis, with insulin playing a leading role as an inducer (2).

The objective of our laboratory is to understand the mechanisms of induction or repression of glucokinase synthesis in molecular terms. Toward this end, we recently isolated a glucokinase cDNA by recombinant DNA techniques and showed that the concentration of glucokinase mRNA in liver increases dramatically over a few hours when starved rats are refed glucose (6). We concluded that the induction of glucokinase in this situation was achieved by a pretranslational mechanism, most probably a stimulation of specific gene transcription.

In the present work, we investigated the molecular mechanism responsible for the induction of glucokinase in diabetic rats after insulin administration. The major question was to determine whether insulin had an acute effect in vivo on the rate of transcription of the glucokinase gene. Such an effect is documented in this paper, together with the resulting time course of accumulation of glucokinase mRNA and, eventually, of enzyme protein.

**EXPERIMENTAL PROCEDURES**

*Animals—Male Wistar rats were purchased from the breeding facility of the University of Geneva School of Medicine and used when they weighed 120–160 g. Diabetes mellitus was induced by intravenous injection of 80 mg of streptozotocin/kg of body weight 8–10 days before the experiments. Only animals with a plasma glucose concentration higher than 25 mmol/L on three separate days were included in the study. The plasma insulin concentration was below 0.3 ng/ml versus 1.3 ng/ml in nondiabetic controls. Highly purified porcine insulin was administered by three intraperitoneal injections of 20 units/kg of body weight at 0, 8, and 16 h, respectively. The total experimental period extended over 24 h. Most of the experiments were started between 8:00 and 9:00 a.m. In one case, animals were adapted to a 3:00 p.m.–3:00 a.m. light cycle, and insulin treatment was started at 4:00 p.m. The animals had free access to food pellets and drinking water. The pellets contained approximately 60% carbohydrate with respect to energy supply. At chosen times, the rats were stunned by a sharp blow to the head and decapitated. The livers were rapidly excised and washed in an ice-cold buffer containing 6 mM Tris/HCl, pH 7.5, and 140 mM NaCl.***
Glucokinase Assay—Pieces of liver weighing 500 mg were minced with scissors and homogenized at 4 °C in Teflon-glass homogenizers containing 2 ml of the homogenization buffer described previously (6). The cytosol fraction of the homogenates was separated by ultracentrifugation and assayed for glucokinase activity as described (6). The protein concentration was measured by the Bradford method (7).

Immunoblotting of Glucokinase with Affinity-purified Antibodies—Cytosol proteins were resolved by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose filters by standard procedures (8, 9). Immunoblotting was performed with affinity-purified rabbit antibodies raised against liver glucokinase purified in a sheep. These antibodies were revealed by rabbit antibodies to sheep immunoglobulins, followed by affinity-purified 125I-labeled Protein A (specific activity 30 mCi/mg of Protein A). Solutions for blocking, antibody incubations, and washing were described previously (10). Size marker proteins were localized on blots by staining with India ink (11).

Affinity purification of the primary antibodies was performed using a galactosidase-glucokinase fusion protein encoded by the recombinant glucokinase cDNA phage λ-GK223, as described in a previous publication (6). Briefly, the fusion protein synthesized by a λ-GK223 lysogen was bound to nitrocellulose by preparative Western transfer. The immobilized protein was then used as an adsorbent to select antibodies specific to glucokinase epitopes from the serum of a sheep immunized with highly purified glucokinase from rat liver.

Isolation of RNA and Northern Blot Analysis—Samples of freshly excised tissue were homogenized with an Ultra-Turrax at 4 °C with 300 μg/ml protease inhibitors in a medium containing 2 ml of the homogenization buffer described previously (6). The run-on transcription reaction in isolated nuclei was carried out at 26 °C for 20 min using the reaction mixture described (17). The ribonuclease digestion step was omitted. The high-speed centrifugation and assay for glucokinase activity were described as with Northern blots.

RESULTS

Time Course of Glucokinase Induction in Liver after Insulin Administration to Diabetic Rats—In earlier reports, the time required for the restoration of normal glucokinase activity in diabetic rats treated with insulin varied between 2 and 24 h (3, 4, 21). Differences in the severity of diabetes, as well as in insulin dosage and route of administration, might account for this discrepancy. It was therefore necessary to establish the time course of glucokinase induction under the precise experimental conditions of the present study. One approach we used to estimate the amount of glucokinase in liver was based on Western transfer of total cytosol protein and immuno blotting, using affinity-purified antibodies to glucokinase (Fig. 1). The amount of glucokinase was shown to be reduced drastically in the livers of diabetic rats. It remained low for 4 h after insulin administration. Subsequently, the amount of enzyme increased in a time-dependent fashion and approached the level seen in normal animals 24 h after the initial dose of insulin.

To corroborate this time course, glucokinase activity in the cytosol fraction of liver homogenates was measured by a conventional spectrophotometric assay (Table I). Glucokinase activity was barely detectable in diabetic rats until 4 h following the administration of insulin. Thereafter, the enzyme activity rose to 25% of the nondiabetic level after 8 h of treatment and to 65% of the normal level 24 h after treatment. The general agreement between results from enzymatic analysis and immunoblotting supports the view that changes in assayable glucokinase activity reflect changes in the amount of enzyme, rather than in the catalytic efficiency of preexisting enzyme. The time course of enzyme induction presented here is in good agreement with the original data from Weinhouse’s and Sols’ laboratories (3, 4). At variance with the more recent report of Spence (21), we never observed rapid increases in glucokinase activity occurring within 2–3 h of insulin administration.
the induction process at the mRNA level. Samples of RNA were subjected to Northern blot analysis, using a cloned glucokinase cDNA probe (6). As shown in Fig. 2, the amount of glucokinase mRNA in liver increased dramatically following insulin administration to diabetic rats. In contrast, the abundance of serum albumin mRNA (Fig. 2, inset) was little affected throughout the period of study, demonstrating the specificity of the hormonal effect on glucokinase. The insulin effect at various times of treatment was quantitated by densitometer tracing of Northern blot autoradiograms (Table I). A clear-cut increase in glucokinase mRNA amount was noted by 1 h after insulin injection. Further accumulation of mRNA occurred until 8 h after the start of treatment. Subsequently, in spite of repeated insulin injections at 8 and 16 h, the level of glucokinase mRNA fell precipitously so that little mRNA was left by 16 h (Fig. 2), and none was detectable 24 h after the initial dose of insulin. It should be emphasized that at its peak level, namely at the 8-h time point of this study, glucokinase mRNA was increased 13-fold above the average level seen in normal rats fed ad libitum. It should be noted further that the abundance of glucokinase mRNA in the normal fed state, which may be considered as the maintenance level for high glucokinase activity, is quite low, only slightly above the quantification limit using the present experimental conditions.

**Rapid Effect of Insulin on Transcription of Glucokinase Gene**—The rapid and massive build-up of glucokinase mRNA after insulin administration suggests an hormonal effect at the transcriptional level. To investigate this point, we performed “run-on” transcription experiments with isolated liver nuclei. In this procedure, nascent RNA transcripts initiated by RNA polymerase in vivo are elongated in vitro in a reaction

**TABLE I**

Time course of insulin effect on glucokinase activity and mRNA amount in liver of diabetic rats

Experimental diabetes was provoked in rats by injection of streptozotocin 10 days prior to the study. Porcine insulin (20 units/kg body weight) was injected intraperitoneally at time 0. Additional injections at 8 and 16 h were given to animals killed at 24 h. The methods for affinity purification of antibodies to glucokinase and immunoblotting of total liver cytosol protein are described under “Experimental Procedures.” The electrophoretic gel was 11% (w/v) in polyacrylamide. The molecular weights of marker proteins (α2-macroglobulin, phosphorylase, glutamate dehydrogenase, lactate dehydrogenase, and soybean anti-trypsin) are given on the left in kilodaltons. The load of protein on each lane was 15 μg. Lanes 1 and 7, no insulin treatment (two distinct animals); lane 2, 1 h after insulin; lanes 3 and 8, 4 h after insulin (two animals); lanes 4 and 9, 8 h after insulin (two animals); lanes 5 and 10, 24 h after insulin (two animals); lane 6, nondiabetic control animal.

| Time after insulin | No. of rats | Glucokinase activity | Relative amount of glucokinase mRNA |
|--------------------|-------------|----------------------|-----------------------------------|
| h                  |             | milliunits/mg protein | arbitrary units                    |
| 0                  | 5           | 0.7 ± 0.5             | 0                                  |
| 1                  | 3           | 0                    | 0.87 ± 0.18                        |
| 4                  | 3           | 1.0 ± 0.5             | 2.78 ± 0.82                        |
| 8                  | 5           | 8.5 ± 1.5             | 5.25 ± 0.86                        |
| 24                 | 5           | 23.2 ± 5.1            | 0                                  |
| Nondiabetic controls | 5         | 35.5 ± 6.6            | 0.43 ± 0.32                        |

**FIG. 1.** Time-dependent accumulation of glucokinase in liver after insulin administration to diabetic rats. The autoradiogram of a Western blot is shown. Experimental diabetes was provoked by injection of streptozotocin 10 days prior to the study. Porcine insulin (20 units/kg body weight) was injected intraperitoneally at time 0. Animals killed at 24 h. The methods for affinity purification of antibodies to glucokinase and immunoblotting of total liver cytosol protein are described under “Experimental Procedures.” The electrophoretic gel was 11% (w/v) in polyacrylamide. The molecular weights of marker proteins (α2-macroglobulin, phosphorylase, glutamate dehydrogenase, lactate dehydrogenase, and soybean anti-trypsin) are given on the left in kilodaltons. The load of protein on each lane was 15 μg. Lanes 1 and 7, no insulin treatment (two distinct animals); lane 2, 1 h after insulin; lanes 3 and 8, 4 h after insulin (two animals); lanes 4 and 9, 8 h after insulin (two animals); lanes 5 and 10, 24 h after insulin (two animals); lane 6, nondiabetic control animal.

**FIG. 2.** Time-dependent accumulation of glucokinase mRNA in liver after insulin administration to diabetic rats. The autoradiogram of a Northern blot is shown. The design of the study is described in Fig. 1. Liver RNA was isolated from the same animals. The procedures for RNA isolation, gel electrophoresis, RNA transfer to a nylon filter and hybridization to 32P-labeled cDNA probes are described under “Experimental Procedures.” The probes were the 1,800-bp glucokinase cDNA of plasmid pUC-GK1 (main picture) and the 1,100-bp albumin cDNA of plasmid pRSA13 (inset). The load of total tissue RNA was 5 μg/lane for the glucokinase cDNA blot (except lane 8, 0.25 μg) and 1 μg for the albumin cDNA blot. The probes had similar specific activity and autoradiography exposure times were 20 and 0.5 h, respectively. Lane 1, nondiabetic control; lane 2, diabetic, no insulin treatment; lane 3, 1 h after insulin; lane 4, 4 h after insulin; lane 5, 8 h after insulin; lane 6, 16 h after insulin; lane 7, 24 h after insulin; lane 8, 8 h after insulin, 20 times less RNA than lane 5.
mixture containing labeled UTP. Specific gene transcripts are isolated subsequently by hybridization to excess complementary DNA sequences immobilized on filters, and precursors incorporation into these transcripts is measured. Incorporation values provide an estimate of the number of RNA polymerase molecules that were engaged in the transcription of the genes of interest at the time of isolation of the nuclei. In the present experiments, the transcripts elongated in vitro were hybridized to a glucokinase cDNA plasmid, as well as to cDNA plasmids encoding serum albumin and the gluconeogenic enzyme phosphoenolpyruvate carboxykinase. Transcription of the serum albumin gene was chosen as reference, because its rate is high in liver and should be little affected by insulin treatment (see above data on albumin mRNA and Ref. 22). The phosphoenolpyruvate carboxykinase gene, on the other hand, was of interest because of its well established negative regulation by insulin in rat hepatoma cells (23). The rate of transcription of these genes was assessed by autoradiography of the plasmid filters (Fig. 3), and quantitation was performed by densitometer scanning of autoradiograms (Table II). As may be seen, the rate of transcription of the glucokinase gene in liver was increased dramatically 45 min after insulin administration to the diabetic rat. Conversely, the transcription rate of the phosphoenolpyruvate carboxykinase gene was strongly repressed. As a result of these opposite changes, the rates of transcription of the two genes became comparable, whereas they were separated by 2 orders of magnitude before insulin administration. Both effects were reversed at 8 h of treatment. The transcriptional activity of the albumin gene remained quasi-stable throughout the study as anticipated.

**DISCUSSION**

The data presented here show that the induction of liver glucokinase in diabetic rats treated with insulin results primarily from a transcriptional effect at the level of the gene. We found that the rate of transcription of the glucokinase gene in liver was increased about 20-fold 45 min after intraperitoneal insulin administration. This effect is remarkably rapid, considering that insulin had to be absorbed from the peritoneal cavity before acting on target cells. As a result of the strong activation of specific gene transcription, glucokinase mRNA accumulated massively in the liver for a period of some 8 h after insulin administration. At the end of this period, the rate of transcription of the gene was back to the prestimulation level, and the amount of glucokinase mRNA started to decline. From the 90% drop in mRNA level occurring between the 8th and 16th h of insulin treatment, we concluded that the half-life of the mRNA was in the order of 2–3 h during this time interval. It is conceivable that a stabilization of glucokinase mRNA contributes to its accumulation during the initial phase of induction, although we have no evidence in support of this hypothesis.

This study reveals a striking, previously unsuspected difference in the temporal patterns of accumulation of glucokinase mRNA and glucokinase enzyme protein in diabetic rats treated with insulin. Whereas glucokinase mRNA was increased markedly as early as 1 h after insulin administration, it took more than 4 h until an increase in glucokinase itself could be detected either by spectrophotometric enzyme analysis or by quantitative immunoblotting. This finding suggests that newly synthesized glucokinase mRNA may not be translated very efficiently early after insulin administration. It is known that the general capacity of the liver for protein synthesis is reduced in the diabetic state and completely restored within 24 h after insulin treatment (24). Such a general effect on the protein synthesis machinery may provide an explanation for the time lag between mRNA build-up and rise in enzyme level.

We noted a sustained increase in glucokinase level between 8 and 24 h after the start of insulin administration concomitantly with a rapid decay of glucokinase mRNA and, presumably, of the enzyme synthesis rate. This implies that glucokinase did not undergo rapid turnover during this time period. The half-life of glucokinase can be estimated easily in chronically diabetic rats withdrawn from insulin treatment, because glucokinase mRNA is essentially absent in these animals and, consequently, the half-life of glucokinase synthesis is virtually nil. Glucokinase activity then decays with a half-time of about 30 h indicating that the enzyme is relatively stable even under deinduction conditions such as diabetes (3). There was a suggestion that the half-life of glucokinase might be increased further during induction by insulin treatment, based on radioimmunochemical data of glucokinase turnover (25). This notion should be considered with caution, however, due to some uncertainty with respect to the specificity of the amino acid incorporation measurements in the concerned study. At any rate, the relatively long half-life of glucokinase allows for sustained increases in glucokinase amount following short pulses of mRNA accumulation, as shown here for diabetic rats treated with insulin.

In earlier work (6, 26), we and others showed that the
induction of glucokinase during the fasting-refeeding transition was also characterized by a massive and equally short-lived accumulation of glucokinase mRNA. Both after a glucose load to fasting animals (6) and after acute insulin administration to diabetic ones (this work), the amount of glucokinase mRNA in liver 6–8 h after the initial stimulus was increased more than 10-fold above the maintenance level seen in freely fed intact rats. What is then the biological significance of control mechanisms entailing such marked overshoots? An obvious advantage of this type of regulation, as compared to smaller and steadier increases in mRNA level and rate of enzyme synthesis, is to shorten the time period required for the transition from low to high enzyme activity. This advantage is especially pronounced for an enzyme like glucokinase, which has a relatively long half-life. Once the enzyme pool has been replenished, a steady state can be maintained with a mRNA content far below the maximally induced level.

Our data raise intriguing questions as to the mechanisms responsible for the down-regulation of glucokinase gene transcription following brief periods of intense transcriptional activity. In the whole animal, the secretion of physiologically antagonistic hormones could contribute to restrain the action of insulin. Furthermore, as yet unknown mechanisms intrinsic to the hepatocyte might operate to ensure that the activation of the glucokinase gene is self-limiting. It will be necessary to perform experiments with cultured liver cells both to identify the individual effectors involved in the control of glucokinase gene transcription and to define their mechanisms of action at the molecular level.

The very rapid burst in the transcriptional activity of the glucokinase gene after insulin administration to the whole animal suggests that insulin itself exerts a direct action on the liver cell. In support of this view, insulin has been shown previously to induce glucokinase activity in cultured hepatocytes (27–29). It is known, however, that the secretion of glucagon by the alpha cell of the islet of Langerhans is suppressed within minutes after insulin administration in diabetic animals (30, 31). We cannot therefore exclude that a fall in the plasma glucagon level was a contributing factor for the stimulation of glucokinase gene transcription in the present in vivo experiments.

The intracellular signalling mechanism used by insulin to control gene activity is not understood. From the present results, it would appear that the glucokinase gene may provide a good model system to investigate this point, especially if the activation of the gene can be reproduced in tissue culture. In this context, it is noteworthy that the induction of transcription of the glucokinase gene and the repression of the phosphoenolpyruvate carboxykinase gene by insulin in diabetic rats occur with similar kinetics. This observation suggests that the two processes may share common intermediary steps. At the DNA level, we hypothesize that one or several specific sequences, termed hormone-response element(s), is (are) present in the vicinity of the glucokinase gene promoter and confer hormone inducibility to it. The identification of these sequences and of putative protein regulatory factors interacting with them might shed new light onto a basic aspect of insulin action.

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