Hormonal Regulation of Cystathionine β-Synthase Expression in Liver*

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Homocysteine metabolism is altered in diabetic patients. Cystathionine β-synthase (CBS), a key enzyme involved in the transsulfuration pathway, which irreversibly converts homocysteine to cysteine, catalyzes the condensation of serine and homocysteine to cystathionine. Studies in streptozotocin-induced diabetic rats have shown that CBS enzyme activity is elevated in the liver but not in the kidney, and this effect is reversed by insulin treatment. To determine whether these effects resulted from alterations at the level of gene transcription, CBS mRNA was measured in diabetic and insulin-treated diabetic rats. CBS mRNA levels were found to be markedly higher in streptozotocin-induced diabetic rat livers; these were reduced by insulin administration. In H4IE cells, a rat hepatoma cell culture model, glucocorticoids increased the cellular levels of CBS enzyme protein and CBS mRNA; insulin inhibited this stimulatory effect. Treatment with insulin also decreased CBS levels in HepG2 cells, a human hepatoma cell line. Nuclear run-on experiments in the rat cells confirmed that stimulation of CBS gene expression by glucocorticoids and the inhibition by insulin occurred at the transcriptional level. Transient transfections of HepG2 cells with a CBS-1b promoter luciferase reporter construct showed that the promoter activity was decreased by 70% after insulin treatment. These results show that insulin has a direct role in regulating homocysteine metabolism. Altered insulin levels in diseases such as diabetes may influence homocysteine metabolism by regulating the hepatic transsulfuration pathway.

Cystathionine β-synthase (CBS)1 (EC 4.2.1.22) catalyzes the first committed step in cysteine biosynthesis, the irreversible condensation of homocysteine with serine to form cystathionine (1). Homocysteine, a sulfur-containing nonprotein amino acid, is an intermediate in the metabolism of methionine. It is at a metabolic crossroads between its synthesis from methionine and its removal through the transsulfuration or remethylation pathways (2). Two pyridoxal 5’-phosphate-dependent enzymes comprise the transsulfuration pathway: CBS, which catalyzes the condensation of serine and homocysteine to cystathionine, and cystathionine γ-lyase, which catalyzes the formation of cysteine, α-ketobutyrate, and ammonia (3, 4). The regulation of CBS gene expression is important in a number of physiological situations. Feeding rats a high protein diet or a high methionine diet increases CBS activity (5). It is also known that flux through the transsulfuration pathway provides cysteine for glutathione synthesis, so that altered CBS levels may be of importance in oxidative stress (6). The well known sparing effect of cysteine on methionine requirements is mediated, at least in part, via alterations in CBS activity. Our own recent work shows that glucagon administration to rats increases hepatic CBS enzyme activity and mRNA levels (7). CBS enzyme deficiency, an autosomal recessively inherited disorder, is the leading cause of homocystinuria. Partial deficiency may lead to hyperhomocysteinemia, causing premature peripheral and cerebral occlusive arterial disease (8, 9).

Elevation of plasma homocysteine levels is recognized as an independent risk factor for the development of cardiovascular disease. The plasma concentration of homocysteine is known to be perturbed in diabetes, being increased when renal insufficiency is evident (10–13). However, patients with insulin-dependent diabetes mellitus with no clinical signs of renal insufficiency, have lower than normal levels of homocysteine (14). Our previous work has shown that the plasma homocysteine level was also decreased in the streptozotocin-induced rat diabetic model with a concomitant increase in hepatic CBS enzyme activity; insulin administration reversed these effects (15). Insulin-dependent diabetes mellitus is characterized not only by insufficient circulating levels of insulin but also by elevated levels of counterregulatory hormones such as glucagon and glucocorticoids (15–17). Moreover, cyclic AMP-elevating agents and glucocorticoids have been shown to increase the level of CBS enzyme activity in rat hepatoma cells (18). It is therefore possible that the increase in the hepatic CBS activity in diabetic rat liver may be brought about by decreased insulin and/or by increased levels of counterregulatory hormones. The aim of this study was to examine the role of insulin and counterregulatory hormones on the expression of the CBS gene, using a diabetic rat liver model as well as rat and human hepatoma cell lines.

EXPERIMENTAL PROCEDURES

Animal Experiments—All procedures were approved by Memorial University’s Institutional Animal Care Committee and were in accord-
ance with the guidelines of the Canadian Council on Animal Care. Male Sprague-Dawley rats weighing 250–350 g were fed laboratory chow ad libitum and had free access to water. Diabetes was induced by a single injection of streptozotocin (100 mg/kg body weight; freshly dissolved in 10 mM citrate buffer (pH 4.5)) through the tail vein, under light ether anesthesia. One hour after the injection, the volume of cells in each rat was adjusted to 2 x 10^7. The streptozotocin-diabetic rats were treated subcutaneously with insulin (Novolin Ultralente human insulin; Lilly) for 5 days to allow the animals to recover from any nonspecific effects of streptozotocin. Thereafter, insulin was withdrawn, and the rats received saline injections for up to 5 days. At this point, insulin was readministered to untreated-diabetic rats for up to 5 days. Control rats received saline throughout the experiment. The insulin (40 units/day) was adjusted to maintain blood glucose close to normal values as measured with an Ames Glucometer II, using a drop of blood obtained by tail prick. On the day of the study, animals were anesthetized with an intraperitoneal injection of sodium pentobarbital (65 mg/kg). Blood samples were taken from the abdominal aorta into heparinized tubes and placed on ice for a few minutes until they were centrifuged at 2700 x g for 15 min, plasma separation. The liver was rapidly removed, freeze-clamped in liquid nitrogen, and stored at −70 °C until used.

Cell Culture—Rat hepatoma H4IIE cells and human hepatoma HepG2 cells were obtained from the American Tissue Culture Collection (Manassas, VA). The CBS-negative a23 Chinese hamster Don fibroblast cell line has been described previously (19). H4IIE cells were grown as monolayers in Dulbecco modified Eagle’s medium containing 10% fetal bovine serum, penicillin (100 units/ml), and streptomycin (100 μg/ml) (Canadian Life Technologies, Burlington, Canada). HepG2 cells and a23 Chinese hamster Don fibroblast cells were cultured in Iscove’s modified Dulbecco’s medium supplemented with fetal bovine serum (10%), penicillin (100 units/ml), and streptomycin (100 μg/ml). Cells were grown to 70–75% confluence under 5% CO2 in a humidified incubator at 37 °C. H4IIE cells were made quiescent by overnight culture in medium containing 0.5% fetal bovine serum. Before the addition of hormones, cells were provided with fresh medium containing 0.5% fetal bovine serum for 1 h. The medium was changed once more before stimulating with triamcinolone and/or insulin (Sigma). At the end of the incubation, media were aspirated, and cells were incubated once more before stimulating with triamcinolone and/or insulin (Sigma). The streptozotocin-diabetic rats were treated subcutaneously with insulin (Novolin Ultralente human insulin; Lilly) for 5 days and then hybridized with a cDNA probe specific for rat CBS (2.3 kb EcoRI fragment of rat CBS cDNA (29), which was random primer-labeled with [α-32P]dCTP (3000 Ci/mmol; PerkinElmer Life Sciences) using the MegaPrime labeling kit (Amersham Biosciences), according to the manufacturer’s instructions. The membranes were stripped and hybridized with a random primer radiolabeled β-actin cDNA probe (CLONTECH Laboratories, Palo Alto, CA) to control for equal loading of RNA and washed at high stringency (final wash, 0.1% SSC, 0.1% SDS at 55 °C). Autoradiography was carried out by exposure of the blot to Kodak XAR or Biomax film. Autoradiographs were scanned on Chemi-Imager™ 4000, and RNA levels were quantitated using the AlphaEase software (Alpha Innotech Corporation, San Leandro, CA).

Reverse Transcriptase-PCR—Total RNA was isolated from H4IIE cells after treatment with hormones as described. 2 μg of RNA was reverse transcribed using a one-step reverse transcription kit (Qiagen, Mississauga, Canada) and amplified by 30 cycles. An upstream primer (5′-GGCTTCAGGACATCCAGTGT-3′) and a downstream primer (5′-TCTTTCCGGGCTTGCCTACGGG-3′) corresponding to the coding sequence of type 3 rat CBS (28) were used to amplify a 1644-bp PCR fragment. A 768-bp fragment of the rat β-actin was co-amplified using amplimer set primers (CLONTECH Laboratories, Palo Alto, CA). PCR products were separated on 0.8% agarose. Ethidium bromide-stained bands were visualized by UV illumination. The gel was imersed in 0.25 M HCl for 30 min, denaturated in denaturing buffer (1.5 M NaCl, 0.5 M NaOH) for 30 min, rinsed with distilled water, soaked in neutralization buffer (0.5 M Tris-HCl, pH 7.0, 1.5 M NaCl) with gentle agitation, incubated in 10× SSC, and transferred to S and S Nytran SuPer charge—nylon membrane. After transfer, the DNA was immobilized by UV cross-linking in a Hoefer UVC 500 UV cross-linker (Amersham Biosciences). The blots were simultaneously probed with 32P-labeled CBS cDNA and radiolabeled β-actin cDNA probe (CLONTECH Laboratories, Palo Alto, CA). Autoradiography was carried out using Kodak XAR or Biomax film.

Recombinant Plasmids, Transfection, and Luciferase Assays—The sequence and construction of the CBS-1b promoter luciferase reporter construct pCBS47 have been described previously (30). The control luciferase reporter constructs pGL-Control (positive control), pGL3-basic (negative control containing no promoter), and pRL-CMV (internal transfection control) were all obtained from Promega. Luciferase transfection was performed using Lipofectin (Invitrogen) according to the manufacturer’s standard protocol for attached cells and typically used 0.5 μg of the test construct and 50 ng of the internal pRL-CMV per well in a 24-well tissue culture plate. After transfection, cells were harvested in situ using direct cell lysis buffer (Promega) and were subsequently assayed for both Renilla and firefly luciferase activities using a dual luciferase system (Promega) and a Moonlight 2010 lumimeter (Analytical Luminescence) according to the manufacturer’s instructions. All promoter assay values were corrected for transfection efficiency by normalizing the firefly luciferase signal of the CBS-1b promoter construct to the Renilla luciferase values derived from the co-transfected, control vector pRL-CMV. All values are expressed as ratios of the test to the internal control reporter expression. Unless otherwise stated, the results shown are the average of at least three independent experiments performed in triplicate, with the error bars representing S.E.

Nuclear Run-on Analysis—Nuclear run-on assays were performed according to a previously described method (31). H4IIE cells were cultured in 150-cm2 flasks, and, for nuclear run-on, the nuclei were isolated from 5 x 10^7 cells. Nuclear suspensions (200 μl) were incubated with 2.5 μM each of CTP, ATP, and GTP and with 200 μl of α-32P-UTP (3000 Ci/mmol; PerkinElmer Life Sciences). The 32P-labeled RNA was isolated, after deoxyribonuclease I and proteinase K treatments, by phenol-chloroform extraction and ethanol precipitation; it was further purified by Micro BioSpin 30 (Bio-Rad) chromatography. Equal amounts (10^4 cpm/ml) of
Effect of diabetes and insulin treatment on plasma homocysteine and plasma glucose in rats

Values are expressed as mean ± S.D. (n = 5). Values with differing numbers of asterisks within each column are significantly different, p < 0.05.

| Rats                  | Plasma homocysteine | Plasma glucose |
|-----------------------|---------------------|----------------|
|                       | µM                  | µM             |
| Control               | 8.8 ± 1.7***        | 7.6 ± 1.6*     |
| Diabetic              | 3.2 ± 0.5**         | 32.2 ± 5.6**   |
| Diabetic + 1 day insulin | 5.5 ± 0.8***       | 9.3 ± 3.5*     |
| Diabetic + 3 days insulin | 5.9 ± 0.7**        | 5.5 ± 1.4*     |
| Diabetic + 5 days insulin | 6.1 ± 2.2**        | 5.9 ± 0.8*     |

Table I gives data on plasma homocysteine and glucose levels in diabetic rats. Plasma glucose levels were markedly higher in streptozotocin-injected rats, indicating profound diabetes. This returned to normal levels on insulin treatment. The animals were also ketogenic. Mean blood pH in diabetic rats was 7.18 ± 0.12, and the sum of β-hydroxybutyrate and acetoacetate was 5.5 ± 0.8 mM. This ketosis was corrected by insulin treatment. Table I shows that plasma homocysteine level in the diabetic rats was only about 40% of that found in control rats. Treatment of diabetic rats with insulin increased plasma homocysteine levels within 1 day of treatment but did not return them to the control level even after 5 days of treatment. To examine whether the lowering of homocysteine levels in the untreated diabetic rat could be attributed to an increase in CBS enzyme activity and, by extension, an increase in homocysteine catabolism through the transsulfuration pathway, we measured the activity of this enzyme in livers from control, diabetic, and insulin-treated diabetic rats. CBS activity was elevated in the diabetic rats; this was returned to control values by insulin treatment (Fig. 1A). To determine whether this observed decrease in CBS enzyme activity is regulated at the level of transcription, we measured CBS mRNA in treated and untreated diabetic rat livers. CBS mRNA levels were higher in diabetic rat livers compared with control rat livers, and this was reversed by insulin treatment (Fig. 1B). When normalized for β-actin mRNA, the level of CBS mRNA was doubled in diabetic rat liver. Insulin treatment decreased the level of CBS mRNA to control values within a day (Fig. 1C).

Insulin-dependent diabetes mellitus is marked not only by a decrease in insulin levels but also by an increase in the circulating levels of the so-called counterregulatory hormones, glucocorticoids and glucagon (16, 17). To evaluate whether insulin and its counter-regulatory hormones exert a direct and specific effect on liver cells, we used hepatoma cell lines. The basal CBS enzyme activity is low in H4IIE cells (rat hepatoma). However, CBS activity was elevated in these cells when stimulated with glucocorticoids. Specifically, the CBS activity in H4IIE cells, stimulated with the glucocorticoid analogue triamcinolone, exhibited an ~1.5-fold increase in CBS enzyme activity, which fell to normal levels when subjected to insulin treatment (Fig. 2A). To examine whether this increase in enzyme activity in response to triamcinolone was due to an increased quantity of CBS protein, total cell lysate proteins were analyzed by Western blot. A significant increase in the level of CBS protein was evident in triamcinolone-stimulated H4IIE cells, and this was dependent on the concentration of triamcinolone. Fig. 2B shows the increase in CBS enzyme protein expression in response to increasing concentrations of triamcinolone. The 63-kDa CBS protein was evident in cells stimulated with 50 nM of triamcinolone, increased in a dose-dependent manner, and reached a maximum at a triamcinolone concentration of 1 µM. H4IIE cells were therefore stimulated by a fixed concentration of triamcinolone (100 nM) in the presence of various doses of insulin (10 nM to 1 µM). Fig. 2C shows that this glucocorticoid-mediated CBS expression was markedly inhibited by insulin at a concentration of 10 nM, and complete inhibition of CBS expression was seen at a concentration of 1 µM. To examine the effects of triamcinolone and insulin on CBS gene expression, total RNA was isolated from hormone-treated and control H4IIE cells. Total RNA (2 µg) was reverse transcribed and amplified as described under “Experimental Procedures.” Amplified products were analyzed by agarose gel electrophoresis. Ethidium bromide staining showed the expected 1642-bp CBS and 764-bp β-actin. Control cells expressed only low levels of CBS mRNA. To confirm that the products seen on ethidium bromide-stained gels were indeed CBS and β-actin, agarose gels were Southern blotted and...
amcinolone (100 nM) in the presence of increasing concentrations of biotinylated molecular weight standards.

From H4IIE cells that had been incubated with 1 nM triamcinolone (10, 50, 100, and 1000 nM); insulin (1, 10, 50, 100, and 1000 nM), respectively; and glucagon showed no effect on CBS mRNA expression. Our results show that triamcinolone and insulin exert opposing effects on CBS enzyme activity, protein concentration and mRNA levels in H4IIE cells. Glucocorticoids are known to increase in cells that had been stimulated with triamcinolone. When normalized to the β-actin transcription rate, there was an approximately 2.5-fold increase in the CBS transcription rate after triamcinolone stimulation. Insulin treatment reversed this increase, suggesting that the effect of both hormones is mainly exerted at the level of transcription (Fig. 4, A and B).

We next asked whether the effects of hormones on CBS enzyme activity and expression seen in rodent cells could be demonstrated in a human cell model. There was a strong basal expression of CBS in nonconfluent HepG2 cells. When these cells were subjected to insulin treatment, CBS enzyme activity was decreased by about 70%. Treatment with the counterregulatory hormone, glucagon, had no effect on CBS enzyme activity in these cells (Fig. 5A). These results were very similar to those obtained from the rat model. Western blotting analysis showed that this decrease in CBS activity is accompanied by decreased levels of CBS protein in insulin-treated cells compared with control. There was no change in the level of CBS protein in glucagon-treated cells (Fig. 5B). To examine whether insulin exerted its effect by controlling the promoter elements that mediate CBS gene transcription, HepG2 cells were transfected with the CBS-1b promoter reporter construct pCBS47 fused to a luciferase reporter gene (30). Treatment of the transfected cells with 0.1 μM insulin for 48 h resulted in a marked decrease in luciferase activity (Fig. 5C), showing that the decreased CBS activity brought about by insulin is mediated through insulin-sensitive sequence(s) on the CBS gene regulatory region. Stimulation by glucagon treatment had no effect on CBS promoter activity.

Regulation of Cystathionine β-Synthase Expression

![Graph of CBS enzyme activity in control, triamcinolone and triamcinolone plus insulin-treated H4IIE cells. Data are expressed as means ± S.D. Differences in letters (a, b) between columns signify significant differences (p < 0.05). B, Western blot analysis of CBS protein expression in H4IIE cells. H4IIE cells were stimulated with increasing concentrations of triamcinolone. Lane 1, control; lanes 2–5, triamcinolone (10, 50, 100, and 1000 nM); lane 6, Me2SO control; lane 7, biotinylated molecular weight standards. C, cells stimulated with triamcinolone (100 nM) in the presence of increasing concentrations of insulin. Lane 1, triamcinolone (100 nM); lanes 2–6, triamcinolone (100 nM) and insulin (1, 10, 50, 100, and 1000 nM), respectively; lane 7, biotinylated molecular weight standards. The blot is typical of three independent experiments.](Image 50x381 to 294x728)

![Figure 3. Reverse transcriptase-PCR analysis of rat CBS mRNA. H4IIE cells treated for 18 h with triamcinolone, CPT cAMP, or glucagon in the presence or absence of insulin. RNA (2 μg) was reverse transcribed, amplified with gene-specific primers, and transferred to nylon membranes. Blots of the PCR products were probed with 32P-labeled CBS cDNA and β-actin cDNA. A, lane 1, control; lane 2, triamcinolone (1 μM); lane 3, triamcinolone (1 μM) plus insulin (1 μM); lane 4, CPT cAMP (200 μM); lane 5, CPT cAMP plus insulin (1 μM); lane 6, glucagon (1 μM); lane 7, glucagon (1 μM) plus insulin (1 μM); lane 8, triamcinolone (1 μM) plus CPT cAMP (200 μM); lane 9, triamcinolone (1 μM) plus CPT cAMP (200 μM) and insulin (1 μM); lane 10, insulin (1 μM). B, densitometric quantitation of CBS mRNA normalized to β-actin. Data presented are mean ± S.D. of 3–5 experiments.](Image 308x485 to 552x728)
DISCUSSION

It is now clear that homocysteine metabolism is impaired in diabetic patients (15, 33). Those with nephropathy have increased plasma homocysteine levels (10, 12, 13). This may be due to the fact that the kidney is an important organ in the removal of homocysteine (33, 34). In contrast, diabetic patients with normal creatinine levels (indicator of kidney function) have lower plasma homocysteine levels (13). The present study shows that plasma homocysteine levels are decreased in the streptozotocin-induced diabetic rat model. Streptozotocin-treated rats were frankly diabetic as indicated by elevated plasma glucose levels, which were well controlled in the insulin-treated diabetic group. We have previously shown that plasma creatinine levels were not elevated in diabetic rats (15). Hence, the changes in plasma homocysteine levels were not due to changes in renal function. Our data show that in diabetic rats there is an increased CBS enzyme activity accompanied by a concomitant increase in CBS mRNA expression, which can be reversed by insulin treatment. Thus, the decreased homocysteine levels in the Type 1 diabetic model are clearly associated with an increase in CBS activity, a key enzyme involved in the catabolism of homocysteine. This, in turn, is regulated by an increase in CBS gene expression, suggesting that the hepatic transsulfuration pathway is regulated by insulin possibly at the level of transcription. Increased flux through the hepatic transsulfuration pathway is also suggested by earlier work from this laboratory, which demonstrated that the hepatic content of cysteine is increased 3-fold in diabetic rats, whereas the level of methionine is significantly lowered (35). This enzyme is also regulated by S-adenosylmethionine, which is a positive allosteric regulator of CBS (36). However, we have measured the levels of this metabolite in freeze-clamped livers and have found no significant change in streptozotocin-diabetic rats with or without insulin treatment (control, 79.1 ± 7.5; diabetic, 75.3 ± 1.5; diabetic + insulin, 80.8 ± 6.9 nmol/g liver). CBS enzyme activity is known to play an important role in determining plasma homocysteine levels. The human CBS gene has been localized to chromosome 21 (21q22.3) and is overexpressed in the trisomy 21 of Down’s patients (37, 38). These patients have lower than normal plasma homocysteine (39). CBS knockout mice have also been produced (40). Heterozygous CBS−/− mice have a 50% reduction in CBS expression and twice the normal homocysteine levels (39). They also exhibit marked impairment in endothelial function (41, 42).
Recent work from Loscalzo’s group (43, 44) has provided strong evidence that the adverse effects of homocysteine are at least partly mediated by oxidative inactivation of nitric oxide (NO). They showed that the impaired endothelium-dependent vasodilation seen in CBS-deficient mice can be attenuated either by the overexpression of glutathione peroxidase or by increased cellular thiols pools (43, 44). Therefore, CBS expression plays an important role in determining plasma homocysteine levels and vascular function. This is confirmed in our study, where diabetes increases CBS activity, insulin treatment decreases it, and both of these changes are reflected in plasma homocysteine levels.

Our data clearly indicate that the effects of glucocorticoids and insulin on CBS expression occur at the transcriptional level. First, changes in enzyme activity are paralleled by changes in the levels of CBS protein and mRNA abundance. Second, in H4IIE cells, nuclear run-on assays clearly showed that stimulation of CBS expression by glucocorticoids and inhibition by insulin occurred at the level of transcription. In addition, actinomycin D inhibited the glucocorticoid-mediated induction of CBS mRNA in H4IIE cells (results not shown). Finally, transfection of a CBS-1b promoter-luciferase construct into HepG2 cells clearly showed that insulin repressed the promoter activity. This reflected the changes in CBS enzyme activity and the relative abundance of CBS protein, indicating a direct regulation of the promoter activity by insulin.

The basal expression of CBS is fairly strong in rat liver cells (15), but in H4IIE cells it was quite weak. In these cells, CBS expression was markedly enhanced by glucocorticoid stimulation, whereas cAMP elicited a moderate stimulation. An earlier report has shown that the level of CBS enzyme activity in H15 and Ad-1 cells (rat hepatoma variants) was elevated when stimulated with a combination of dexamethasone and cyclic AMP (18). However, the molecular control of CBS gene expression was not investigated. Our results indicate that CBS enzyme protein and mRNA can be induced by triamcinolone alone in H4IIE cells. Stimulation of H4IIE and HepG2 cells by glucocorticoids, and Ad-1 cells (rat hepatoma variants) was elevated when stimulated with a combination of dexamethasone and cyclic AMP (18).

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