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Partial Reversal of α2u Globulin Gene Expression by Thyroxine in the Liver of Diabetic Rats†

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ABSTRACT: Synthesis of α2u globulin and its mRNA has been used as an index to monitor the effect of thyroxine on specific gene expression in the liver of hypoinsulinemic male rats. Administration of a physiological dose of thyroxine can partially reverse (to approximately 30% of the normal control) the marked reduction (more than 90%) in the hepatic levels of α2u globulin and its mRNA during streptozotocin-induced diabetes. Estimation of newly synthesized α2u globulin RNA transcripts from the native chromatin of isolated liver nuclei by "nuclear runoff experiments" showed that thyroxine can elevate the rate of transcription of α2u globulin gene in the diabetic rat. Hypoinsulinemic diabetes is also found to be associated with an approximately 35% reduction in the thyroid hormone receptor level as compared to the normal control. The stimulatory effect of thyroxine on the synthesis of α2u globulin and its mRNA was also evident in spontaneous diabetic Wistar "BB" rats. From these studies it can be concluded that severe hypoinsulinemia can cause a decrease in thyroid hormone action at the level of specific gene expression.

Liver is an important target organ for both insulin and thyroxine. An optimum hepatic synthesis of the major urinary protein α2u globulin in the rat is known to require both of these hormones (Roy et al., 1976, 1980; Kurtz et al., 1976). In addition, androgens, glucocorticoids, and growth hormone are also involved in the regulation of α2u globulin and its mRNA (Roy et al., 1983). The multihormonal control of α2u globulin thus serves as a useful model for the study of hormonal interactions at both the systemic and cellular levels in the regulation of specific gene expression.

A body of clinical and experimental results suggest physiological interaction between thyroid hormone and insulin in the regulation of target cell function. Thus hypoinsulinemic diabetes, both in humans and in experimental animals, is frequently associated with an impaired thyroid function. This impairment is believed to be mediated through a decreased availability of the thyrotropin release factor (TRH) and a reduction in the extra thyroidal deiodination of T_4 (Gonzalez et al., 1980; Chopra et al., 1981; Wiersinga et al., 1982). In order to understand the biochemical basis of the interaction between insulin and thyroid hormone, we have examined the role of thyroxine in the reversal of insulin deficiency with respect to the synthesis of α2u globulin and its mRNA in the diabetic rat.

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Studies involving chemically induced diabetes are subject to the limitations associated with the cytotoxicity of chemicals like alloxan and streptozotocin on various tissues including the liver (Rerup, 1970). Toward this end, spontaneous diabetic Wistar "BB" rats provide a useful animal model. These rats develop a genetically predisposed insulin-dependent nonobese diabetic state that closely approximates insulin-dependent juvenile diabetes in the human. The onset of diabetes in these animals is 60–120 days of age. Animals are lean and require daily insulin injection for survival (Like et al., 1982). To rule out any ambiguous interpretations of our results, we have studied the effect of thyroxine both on streptozotocin-induced diabetes and on genetically predisposed diabetic BB rats. Results show that the drastic reduction in the synthesis of α2u globulin and its mRNA observed in hypoinsulinemic diabetes can be partially prevented by thyroxine alone.

Materials and Methods

Animals and Treatment. Experiments were performed either on male Sprague-Dawley rats (300–350 g) treated with streptozotocin or on BB rats (Sir Frederick W. Banting Research Center, Ottawa, Canada) that had developed diabetes (around 80 days of age). The animals were housed in an air-conditioned room (22 ± 1 °C) with 12 h of light and darkness. The control and experimental animals were pair-fed Purina Rat Chow (Ralston Purina Co., St. Louis, MO) and had access to tap water ad libitum. For the chemical induction of diabetes, a single injection of streptozotocin (4.5 mg/100 g of body weight, in citrate buffer, pH 4.5) was given to 80-day-old male rats after 16 h of fasting. The state of diabetes was monitored by periodic testing of glycosuria with "test tape" (Eli Lilly & Co., Indianapolis, IN) and of the fasting blood glucose level. Only those animals that showed continued glycosuria with a blood glucose level above 300 mg/dL (on day 4 after streptozotocin injection) were included in the experimental group. Insulin (porcine, purified single component, 0.1 unit/μL, Eli Lilly, Indianapolis, IN) was subcutaneously administered twice daily (0.25 unit per injection) for 7 days. Thyroxine (T_4) (Sigma, St. Louis, MO) was administered intraperitoneally once daily for a week (1.5 μg/100 g, in 0.5 × 10^{-3} mmol of NaOH).

Assay of Hepatic α2u Globulin. Hepatic concentrations of α2u globulin were determined by the double-antibody radioimmunoassay as described earlier (Roy, 1977).

Assay of Nuclear T_3 Receptor. Nuclear T_3 receptor activity was assayed according to a published procedure (Spindler et al., 1975). Briefly, nuclei, after their isolation from liver homogenate, were suspended in 20 mM tris(hydroxymethyl)aminomethane (Tris), 0.25 M sucrose, 1 mM $MgCl_2$, and 5% glycerol (v/v), pH 7.6. For estimation of T_3 binding, 15 pg of ^{125}I-labeled T_3 was incubated with 0.2 mL of nuclear suspension for 1 h at 22 °C in the presence of 20 mM Tris, 0.25 M sucrose, 1 mM $MgCl_2$, 2 mM ethylenediaminetetraacetic acid (EDTA), 50 mM NaCl, and 5% glycerol (v/v) at pH 7.6. The incubation mixture was subsequently chilled, and 2 mL of nuclei suspending solution (as described above) containing 0.5% Triton X-100 was added. The nuclear pellet was recovered by centrifugation at 4 °C for 15 min at 1250*g* and counted in a γ counter. Specific binding was calculated by taking into account the amount of nuclear-bound T_3 radioactivity when incubation was done in the presence of a vast excess (10^{-5} M) of cold T_3. Scatchard plots were prepared from bound and free radioactivity obtained in the absence and presence of increasing quantities of nonradioactive T_3. The DNA content of the nuclear suspension was determined according to the procedure of Webb and Levy (1955).

Measurement of Rate of Synthesis of α2u Globulin in Rat Liver. Hepatic proteins were pulse labeled in vivo for 18 min by intraperitoneal injections of [^{35}S]methionine (1300 Ci/mol, New England Nuclear, Boston, MA). The amount of α2u globulin synthesized within 18 min was quantitated by specific immunoprecipitation of this protein from the hepatic cytosol, followed by polyacrylamide–sodium dodecyl sulfate (SDS) gel electrophoresis as described earlier (Roy et al., 1977). The ratio of ^{35}S radioactivity under the α2u globulin peak to total input ^{35}S protein radioactivity was used to estimate the rate of synthesis of α2u globulin.

Isolation and Translation of Hepatic mRNA. Total hepatic nucleic acid was extracted with phenol and SDS. Poly(A)-containing messenger RNAs were isolated by affinity chromatography on oligo(dT)–cellulose (Aviv & Leder, 1972). Poly(A)+ mRNAs were translated (800 ng of RNA/30 μL of reaction mixture) in the nuclease-treated rabbit reticulocyte lysate by using [^{35}S]methionine (1300 Ci/mmol) as the labeled amino acid (Pelham & Jackson, 1976). Translationally active α2u globulin mRNAs were quantitated by immunoprecipitation of α2u globulin from the total translation products, followed by SDS–polyacrylamide disc gel electrophoresis of the immunoprecipitate and estimation of the α2u globulin radioactivity as the percent of total radioactive proteins.

Dot–Blot Analysis of α2u mRNA Sequences. Serially diluted poly(A)+ RNA solutions containing 6 × SSC (1 × SSC = 0.15 M NaCl, 0.015 M sodium citrate) and 7.4% formaldehyde were spotted on a nitrocellulose filter (Thomas, 1980). The filter was baked at 80 °C in vacuo and then hybridized to the ^{32}P-labeled α2u globulin cDNA probe. The probe used for this study was derived from a recombinant pBR322 containing α2u globulin cDNA insert (Roy et al., 1982). α2u globulin cDNA insert was excised with *Hha*I and was labeled by nick translation (Rigby et al., 1977). After hybridization the filter was extensively washed, dried, and exposed to the Kodak AR-5 X-ray film. The dot blots were quantitated by scanning through a densitometer (Cliniscan, Helena Laboratories, Beaumont, TX), and only the autoradiographic spots of the serially diluted RNA corresponding to the linear density range were used for computation.

Estimation of the Rate of Transcription of Specific α2u Globulin Gene by "Nuclear Runoff" Experiments. Isolated liver nuclei were incubated in the presence of [α-^{32}P]UTP (2000 Ci/mmol) and the other three ribonucleoside triphosphates (McKnight & Palmiter, 1979). The ^{32}P transcripts were purified according to Chirgwin et al. (1979). The ^{32}P-labeled total nuclear RNAs were then hybridized to the α2u globulin cDNA containing recombinant plasmid (Roy et al., 1982) immobilized on nitrocellulose filters. The bound radioactivity was counted following its elution with NaOH (McKnight & Palmiter, 1979), and the ratio of bound to total input radioactivity was used as an index of the rate of transcription of the α2u globulin gene. [α-^{32}P]UTP incorporation under the assay condition is >90% sensitive to α-amanitin (1 μg/mL).

Results

Hepatic synthesis of α2u globulin as measured by in vivo pulse labeling shows that streptozotocin diabetes leads to an almost complete disappearance of this protein (Figure 1). Treatment of the diabetic rats for 7 days either with insulin or with thyroxine resulted in increased α2u globulin synthesis, as indicated by incorporation of [^{35}S]methionine into the immunoprecipitated protein. Insulin treatment was about 3 times more effective than thyroxine in reversing the decreased synthesis of α2u globulin.

Table I: α2u Globulin Nuclear Gene Activities and Hepatic α2u Globulin Protein Concentrations[a]

hormonal status of the animal (male)	α2u globulin gene transcription (sp hybridizable cpm per 10^6 cpm of total RNA transcripts)	% normal male	hepatic α2u globulin (ng/mg of total protein)	% normal male
normal	180 (218.4, 141.6)	100	1100 (1329, 871.1)	100
diabetic + vehicle	8.33 (6.66, 10)	4.6	50 (65, 35)	4.5
diabetic + thyroxine	49.03 (58.16, 39.9)	27.2	295 (300.1, 290)	26.8
diabetic + insulin	166.25 (208.5, 124)	92.3	985 (1200, 770)	89.6

[a] Summary of the data comparing the transcription of the α2u globulin gene in the nucleus and the hepatic level of this protein under various endocrine conditions. Averages from two experiments are used to compute percent normal male, and individual values are given within parenthesis.

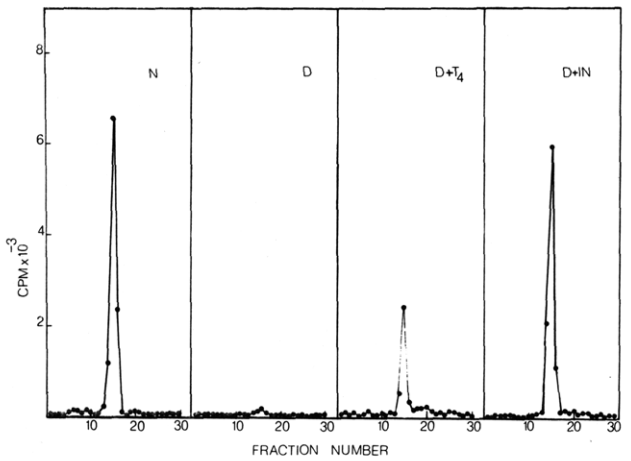

FIGURE 1: Polyacrylamide gel electrophoretic profile of pulse-labeled α2u globulin. Synthesis of α2u globulin was monitored by labeling hepatic proteins with an 18-min pulse of [^{35}S]methionine (0.5 mCi/animal). α2u globulin was quantitatively immunoprecipitated from liver cytosol containing 3×10^5 cpm of protein radioactivity. Immunoprecipitated α2u globulin was electrophoretically separated on SDS–polyacrylamide gel, and the percentage of the total protein radioactivity under the α2u globulin peak was used to estimate in vivo synthesis of α2u globulin for three different endocrine conditions. Abbreviations: N, normal male; D, vehicle-treated diabetic rats (α2u globulin was almost undetectable); D + T_4, diabetic rats treated daily for 7 days with thyroxin (α2u globulin was 30% of that in normal male); D + IN, diabetic rats treated for 7 days with insulin (α2u globulin was 90% of that in normal male). Livers from three rats were pooled for each type of experiment.

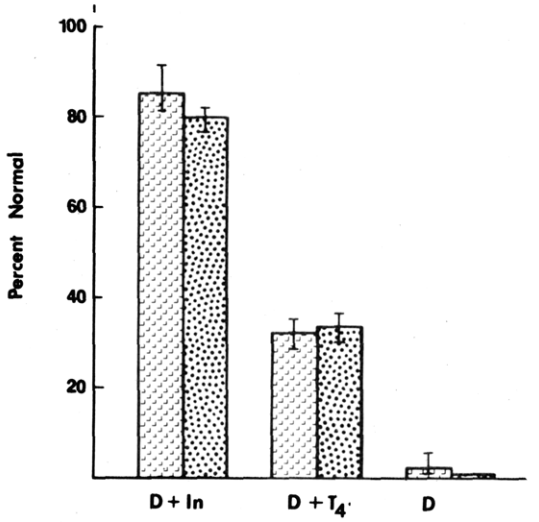

FIGURE 2: Hepatic contents of α2u globulin and translatable mRNA for this protein in diabetic rats after treatment with either insulin or thyroxine. Bar graphs filled with dots represent α2u globulin mRNA quantified through translational assay, and the bars filled with angular signs represent cytoplasmic levels of α2u globulin determined by radioimmunoassay. Abbreviations: D, diabetic treated with vehicle; D + T_4, diabetic treated with thyroxine; D + In, diabetic treated with insulin. Results are expressed as percentage of the normal control male rats of approximately same age (80 days). Each bar graph is a mean of five individual animal experiments (±SD).

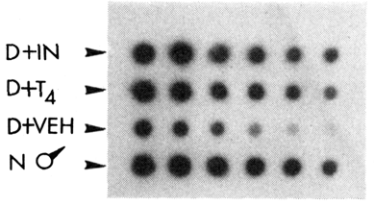

FIGURE 3: Quantitation of α2u globulin mRNA by dot–blot hybridization. Total poly(A)+ hepatic mRNA (2 μg), pooled from three animals, was serially diluted (1:1) and fixed on a nitrocellulose filter. The filter paper was subsequently hybridized with labeled α2u globulin cDNA probe and autoradiographed. Abbreviations: N, normal male; D + VEH, diabetic with vehicle treatment; D + T_4, diabetic treated with thyroxine; D + IN, diabetic treated with insulin. Densitometric scanning of the autoradiographic dots shows that D + VEH, D + T_4, and D + IN represent 6%, 42%, and 74%, respectively, of the α2u globulin mRNA present in the normal male.

In vitro translation of total hepatic mRNA followed by specific immunoprecipitation of α2u globulin showed that the decreased hepatic synthesis of α2u globulin in diabetes and its stimulation either by thyroxine or by insulin are correlated with corresponding changes in the translatable mRNAs for this protein. Figure 2 represents histograms of amounts of functional α2u globulin mRNAs and levels of radioimmunoassayable hepatic α2u globulin in streptozotocin diabetic rats treated with vehicle, thyroxine, or insulin. Increased amounts of translatable α2u globulin mRNAs in diabetic rats after thyroxine supplementation also represent changes in the mRNA sequences as shown by the dot–blot analysis with the cloned cDNA probe for α2u globulin (Figure 3).

Both translational and dot–blot analyses estimate the steady-state level of mRNA populations and do not provide any information concerning the possible changes due to mRNA turnover. In order to examine this question, we have performed nuclear runoff experiments that estimate the rate of transcription of unfinished α2u globulin chains on the chromatin. In these experiments, RNA polymerase II mediated RNA synthesis is initiated on isolated nuclei in the presence of four ribonucleoside triphosphates of which one is radiolabeled ([α-^{32}P]UTP). Since no reinitiation of RNA synthesis occurs under these conditions, the amount of radioactivity incorporated into specific transcripts as compared to the radioactivity in total RNA transcripts gives a measure of the relative rate of transcription of a specific gene. Results of such studies show that the decreased synthesis of α2u globulin in diabetes and the stimulatory effect of insulin and thyroxine are in fact due to alterations in the rate of synthesis of the α2u globulin gene transcripts (Table I). In streptozotocin-induced diabetic male rats, the rate of transcription of α2u globulin gene was only 4.6% of the normal control. Thyroxine supplementation increased the specific transcription rate to 27.2% whereas insulin treatment reversed the inhibitory

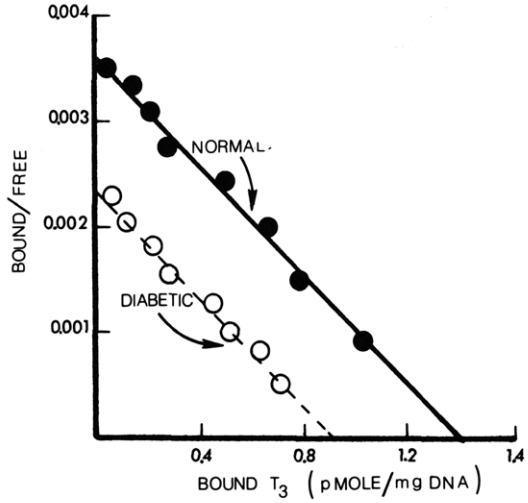

FIGURE 4: Scatchard analysis of nuclear T_3 receptor from normal and diabetic rats. Diabetic rats show about 35% reduction in the specific binding of T_3.

Table II: Hepatic α2u Globulin Levels in Genetically Diabetic BB Male Rats under Different Hormone Therapies[a]

animal type	radioimmunoassayable α2u globulin (ng/mg of total protein)	% normal male
normal BB (nondiabetic)	1015 (3)	100
diabetic + vehicle	85 (4)	8.3
diabetic + T_4	315.3 (6)	31.06
diabetic + insulin	910 (5)	89.6

[a] Data represent the average values from several animals (number of animals used for a particular study is indicated in parenthesis).

effect of diabetes by more than 90%.

Although it has been reported that diabetes impairs the TRH–TSH system, the effect of insulin deficiency on thyroid hormone action at the target cell level through a decreased level of thyroxine deiodinase and the nuclear T_3 receptor has also been suggested (Wiersinga et al., 1982; Chopra et al., 1981). We have therefore looked into the levels of nuclear T_3 receptor in the livers of diabetic rats. Results presented in Figure 4 show about 35% reduction in the nuclear T_3 receptor in diabetic rats. These results are in agreement with those of Wiersinga et al. (1982) and suggest that in experimental diabetes, in addition to systemic changes in the thyroid function, part of the defect may be mediated at the target cell level through changes in the nuclear T_3 receptor concentration.

To ensure that the possible toxic effect of streptozotocin has not influenced the interpretation of our results presented above, we investigated the effect of thyroid hormone in genetically diabetic BB rats. Diabetic BB male rats have 8.3% of the amount of α2u globulin found in nondiabetic control male BB rats. As shown in Table II, thyroxine supplementation increased the hepatic level of α2u globulin to about 30% of the normal control. Insulin was again found to be effective by about 90%. The increased hepatic protein level in these animals reflects the steady-state level of α2u globulin mRNAs as is apparent from the RNA dot–blot analysis (Figure 5). These results suggest that the observed effect of thyroxine is a counterbalance of the endocrinological rather than toxicological influence of the diabetogenic drug used in our studies.

Discussion

Results presented in this article show that the reduced synthesis of α2u globulin in diabetic rats can be partially reversed by thyroxine supplementation. Analyses of both the steady-state level of α2u globulin mRNA and the rate of transcription of α2u globulin gene indicate that the effect of thyroxine is mediated via a corresponding stimulation in the synthesis of α2u globulin mRNA.

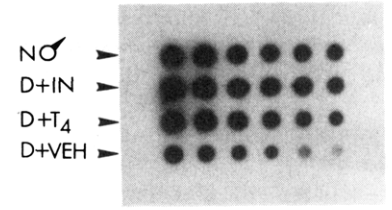

FIGURE 5: Total α2u mRNA sequences in diabetic BB rats before and after hormone therapy as revealed by the dot–blot analysis. The poly(A)+ RNA, (2 μg), pooled from three animals, was serially diluted (1:1). Abbreviations: D, diabetic + vehicle; D + T_4, diabetic + thyroxine; D + IN, diabetic + insulin; N(BB), normal nondiabetic male BB rats. Densitometric scanning of the autoradiogram shows that the amounts of α2u globulin RNA in D, D + T_4, and D + IN animal types represent 4.5%, 46%, and 90%, respectively, of that in the normal male.

Previous studies from several laboratories have reported a decreased level of circulating thyroid hormone associated with diabetes (Gonzalez et al., 1980; Wiersinga et al., 1982; Sundaresan et al., 1984). Clinically low T_3 syndrome in insulin-dependent diabetes mellitus has also been reported (Saunders et al., 1978; Pittman et al., 1979a,b; Naeije et al., 1978). These studies have suggested that impaired thyroid function in diabetes may be mediated both at the systemic level via the hypothalamic–pituitary–thyroid axis and at the target cell level through the diminished nuclear T_3 receptor content and/or thyroxine deiodinase activity. Our results show that such a dual control may be operative in the regulation of α2u globulin and its mRNA in diabetic rats. It is pertinent to note that in some instances thyroid hormone can completely reverse the effect of insulin deficiency of diabetes (Sundaresan et al., 1984). However, examples are also known where the physiological T_3 replacement dose is ineffective in correcting the biochemical alterations observed in diabetes. Sudaresan et al. (1984) have reported that the lowered thyroid hormone level in diabetes mellitus can totally account for decreased myocardial β-adrenergic receptors in streptozotocin-induced diabetes. The lowered number of β-adrenergic receptors in the heart of either thyroidectomized or streptozotocin-induced diabetic rats can be elevated to the normal control level by thyroxine supplementation. When thyroidectomized rats were made diabetic by streptozotocin injection, no additional drop in the receptor number over that in the thyroid-deprived rat was observed. Moreover, insulin supplementation to these rats was ineffective in elevating the receptor level. On the contrary, in the case of myosin heavy chain mRNAs of the rat heart, a multihormonal control involving both thyroid hormone and insulin appears to exist (Dillman, 1982; Dillman et al., 1984). Diabetes and hypothyroidism both result in an elevated level of myosin heavy chain β (MHC β) mRNAs and a lowered level of MHC α mRNAs as compared to age-matched control animals. Although totally effective in hypothyroid rats, the physiological T_3 replacement dose did not normalize the myosin isoenzyme distribution in diabetic rats (Dillman et al., 1984). These variations may reflect differences in the degree of thyroidal contribution in the control of these proteins as well as possible overlaps between thyroxine and insulin action at the molecular level.

Both in vivo and in vitro studies have established that streptozotocin treatment and resulting hyperglycemia increase the synthesis and release of pancreatic somatostatin (Patel & Weir, 1976). Diabetes is also believed to cause general

structural alterations in the hypothalamic neurosecretory cells (Rabkina, 1964). These effects are expected to reduce the hypophysical secretion of growth hormone in diabetic rats. In addition, thyroid hormones are known to stimulate the synthesis and secretion of growth hormone from the pituitary (Hervas et al., 1975; Martial et al., 1977). Interestingly, more than a 90% reduction of α2u globulin mRNAs in thyroidectomized rats can be almost totally reversed by growth hormone supplementation, although thyroid hormone appears to be necessary for the full recovery of the protein level (Chatterjee et al., 1983). The effect of T_3 on a few other T_3-responsive mRNA species in the liver has also been shown to be mediated indirectly through the pituitary production of growth hormone (Liaw et al., 1983). Therefore, a complex interaction between insulin, thyroxine, and growth hormone seems to be operative in the regulation of hepatic gene expression during diabetes mellitus.

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