Diabetes-Induced Alterations in the Translational Activity of Specific Messenger Ribonucleic Acids Isolated from Rat Hearts

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SUMMARY. During diabetes mellitus, total proteins and ribonucleic acids are significantly decreased in the rat heart, and these parameters can be increased by insulin administration. To determine whether all ribonucleic acids are equally sensitive to insulin, we examined the influence of this hormone on individual translatable ribonucleic acids. Cardiac ribonucleic acid prepared from control, untreated, and insulin-treated diabetic animals was translated in vitro in the presence of [3S]methionine. The radiolabeled peptides were separated by two-dimensional gel electrophoresis and were analyzed by fluorometry. We found that diabetes induces both qualitative and quantitative changes in the predominance of a few specific translatable messenger ribonucleic acid species. The translation of 11 messenger ribonucleic acid species was significantly decreased and that of eight messenger ribonucleic acid species was significantly increased in diabetic preparations. Twelve of the 19 translation products were quantified by digital matrix photometry: three labeled peptides were observed only when cardiac ribonucleic acid from diabetic animals was added to the cell-free translation system, four new peptides appeared when cardiac ribonucleic acid from control animals was added, and although the remaining five peptides were translated in vitro after either control or diabetic ribonucleic acid was added, their relative predominance was altered 2- to 200-fold. When translation products coded for by messenger ribonucleic acids prepared from either diabetic or hypothyroid hearts were compared, we found that most of the alterations induced by diabetes were also induced by hypothyroidism. However, two of the 19 products were exclusively regulated by insulin, whereas two products unchanged in the diabetic heart were regulated exclusively by thyroid hormones. Thus, although each hormone specifically influenced the abundance of a few translatable messenger ribonucleic acids, most hormone-sensitive translatable messenger ribonucleic acids are under the dual regulation of insulin and thyroid hormone. (Circ Res 57: 296-303, 1985)

DIABETES induces marked alterations in the cardiac function of animals and humans (Regan et al., 1977; Miller, 1979; Fein et al., 1980). Diabetic rats show a decrease in the maximum velocity of ventricular contraction and a decreased rate of ventricular relaxation (Fein et al., 1980). These pathophysiological conditions return to control levels upon administration of insulin to diabetic animals (Fein et al., 1981). The decreased rate of ventricular contraction observed in insulin-deficient animals has been related to the activity of the Ca++-dependent myosin adenosine triphosphatase (ATPase) (Barany, 1967; Delcayre et al., 1975). Three isoforms of myosin designated V1, V2, and V3 are present in the rat cardiac ventricle (Hoh et al., 1978; Dillmann, 1980; Lompre et al., 1981). The low Ca++-dependent myosin ATPase V3 is the predominant form in diabetic hearts (Dillmann, 1980), whereas V1 is the predominant form found in young, healthy animals (Dillmann, 1980; Lompre et al., 1981; Watras, 1981). V1 and V3 differ by the nature of myosin heavy chains (MHC) present in the native myosin: two MHC-α are present in V1 and two MHC-β are present in V3.

It has been shown recently that two different messenger ribonucleic acids (mRNA) code for these two myosin heavy chains (Mahdavi et al., 1982; Shina et al., 1982; Dillmann et al., 1984), and that the expression of these two ribonucleic acids (RNA) is similarly regulated by insulin and thyroid hormone (Dillmann et al., 1984).

Total protein synthesis is affected by insulin in the heart (Pain et al., 1974; Williams et al., 1980), as well as in noncardiac tissues (DePhilip et al., 1980; Hill et al., 1981; Buse et al., 1984). By using RNA translation in cell-free systems or RNA hybridization to specific deoxyribonucleic acid (DNA) probes, it has been possible to demonstrate that this regulation occurs at a pretranslational level. Thus, the levels of translatable or hybridizable RNA coding for tyrosine aminotransferase (Hill et al., 1980), glucokinase (Spence, 1983; Sibrowski and Setiz, 1984), pyruvate kinase (Noguchi et al., 1982; Municlo et al., 1984), albumin and other plasma proteins (Jefferson et al., 1983), malic enzyme (Drake et al.,...
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The hearts of animals exhibited lower RNA:DNA ratios than normal

groups; group 1 was kept hypothyroid for an additional 4

weeks. Hearts isolated from either diabetic or hypothyroid

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groups; group 1 received daily intraperitoneal injections

of zinc insulin/100 g of body weight, for 3 weeks. Control

animals matched for age were included in the study. Their

blood glucose levels were 125 ± 9 mg/dl. Hypothyroidism

was induced and documented as previously described

(Dillmann et al., 1983). We found that a total

of 19 translation products were affected by diabetes, and

13 of these products could be adequately quantified by digital matrix photometry. In addition, diabetes-induced changes in the level of specific cardiac RNA translation products were compared with those occurring in the hypothyroid rat heart. Only two products were exclusively influenced by diabetes, whereas 12 products were similarly affected by hypothyroidism and diabetes. Thus, the expression of most hormone-responsive mRNA, measured by quantification of translation products synthesized from RNA of normal and hormone-deficient hearts

We quantified the hormone-sensitive spots isolated by two-dimensional gel electrophoresis as previously described (Dillmann et al., 1983), with the aid of a computerized photodiode scanning device developed by Neeley et al. (1981). The integrated density of spot 118, whose predominance was not affected by the hormonal state of the animals, was used to calculate corrected integrated densities as indicated:

\[
\frac{R}{C_v} = \frac{R}{C_v}^{\text{exp}}
\]

where \( R \) = integrated density of hormone-responsive spot, \( C \) = integrated density of spot 118 from the same fluorogram, \( N \) = normal fluorogram, and \( \text{Exp} \) = hormone-deficient fluorogram.

We measured the molecular weight and pI of each spot, as previously described, and used the spot-numbering system described by Dillmann et al. (1983) in all fluorograms.

Statistical Analysis

Corrected integrated densities of identical products measured from each group were compared by two-tailed Student’s t-test.

Results

Figure 1 shows representative two-dimensional patterns of translation products coded for by cardiac RNA isolated from control, diabetic, and insulin-treated diabetic animals. The relative amount of translatable RNA present in total RNA appeared not to be affected by diabetes, since the same amount of total RNA (150–200 μg/ml) was required to saturate the modified rabbit reticulocyte lysate system (data not shown). The abundance of a few specific translatable RNA, however, was consistently affected by the diabetic state. The predominance of eight translation products was significantly in-

Methods

Animal Preparations

Male Sprague-Dawley rats weighing between 200 and 250 g were used in all experiments. We induced diabetes by the administration of 65 mg of streptozotocin/kg body weight. Three days after injection, blood glucose levels were measured and found to average 435 ± 30 mg/dl; they remained constant during the course of the experiments in animals kept without insulin therapy. Three days after diabetes induction, half of the animals began to receive daily subcutaneous injections of 2 U of protamine zinc insulin/100 g of body weight, for 3 weeks. Control animals matched for age were included in the study. Their blood glucose levels were 125 ± 9 mg/dl. Hypothyroidism was induced and documented as previously described (Dillmann et al., 1983). Four weeks after the onset of hypothyroidism, the animals were divided into two groups; group 1 was kept hypothyroid for an additional 4 weeks and group 2 received daily intraperitoneal injections of 0.4 μg of triiodothyronine/100 g of body weight for 4 weeks. Hearts isolated from either diabetic or hypothyroid animals exhibited lower RNA:DNA ratios than normal hearts.

Cell-Free Translation and Analysis of in Vitro Translation Products

Cardiac RNA prepared as previously described (Dillmann et al., 1983) was translated in the modified rabbit reticulocyte lysate (Pelham, and Jackson, 1976). Incorporation of methionine was determined as a function of RNA concentration for each batch of RNA, and nonsaturating concentrations of total RNA were used in subsequent translational assays. An 8- to 15-fold increase in [³⁵S]-methionine incorporation into acid-insoluble material was observed after the addition of exogenous RNA.

Samples containing equal amounts of radioactivity (250–300,000 counts/min) were subjected to two-dimensional gel electrophoresis (Dillmann et al., 1983). After electrophoresis, gels were prepared for fluorography, and were exposed to Kodak XAR-5 films at −80°C. All chemicals used in these experiments were of reagent grade and were purchased from Bio-Rad or Sigma, except for the enzymes used for RNA and lysate preparations, which were purchased from Boehringer-Mannheim Biochemicals, and [³⁵S]methionine, which was purchased from Amersham.

Quantification of Translation Products Synthesized from RNA of Normal and Hormone-Deficient Hearts

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1983) and fatty acid synthetase (Joshi and Aranda, 1979) in the liver, casein in the mammary gland (Hobbs et al., 1982), glycerophosphate dehydrogenase in adipocytes (Spiegelman et al., 1983), and an isoform of pancreatic amylase (Dranginis et al., 1984) all are increased by insulin, whereas the levels of liver phosphoenolpyruvate carboxykinase mRNA are decreased by insulin (Yoo-Warren et al., 1981; Sasaki et al., 1984). Since cardiac physiological functions not related to changes in myosin ATPase are affected by diabetes, it was of interest to determine whether cardiac mRNA other than myosin heavy chain RNA were under insulin influence. Total cardiac RNA isolated from control, diabetic, and insulin-treated diabetic animals was translated in vitro, and the translated products were analyzed by two-dimensional gel electrophoresis as previously described (Dillmann et al., 1983). We found that a total of 19 translation products were affected by diabetes, and 13 of these products could be adequately quantified by digital matrix photometry. In addition, diabetes-induced changes in the level of specific cardiac RNA translation products were compared with those occurring in the hypothyroid rat heart. Only two products were exclusively influenced by diabetes, whereas 12 products were similarly affected by hypothyroidism and diabetes. Thus, the expression of most hormone-responsive mRNA, measured by their ability to directly protein synthesis in vitro, appears to be regulated, directly or indirectly, by more than one hormone. Multihormonal regulation of a few mRNA species has been observed in a wide variety of tissues (Joshi and Aranda, 1979; Hill et al., 1981; Ivarie et al., 1981; Yoo-Warren et al., 1981; Hobbs et al., 1982; Liaw et al., 1983; White and Bancroft, 1983; Winberry et al., 1983, Munnich et al., 1984; Mueckler et al., 1984; Carr et al., 1984).
increased, whereas that of 11 products was significantly decreased in the absence of insulin (Fig. 1). Products 65, 245, and 361 were decreased 2-, 48-, and 87-fold, respectively, whereas products 71, 72, 246, 256, and those indicated by arrows in Figure 1, A and C, could not be detected at all among products translated by cardiac RNA from diabetic animals (Fig. 1B). Inversely, the predominance of eight products was increased in the absence of insulin: products 41, 97, and 106 were increased 3-, 141-, and
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Relative Predominance of Translation Products Responsive to Insulin or Thyroid Hormone

<table>
<thead>
<tr>
<th>Spot</th>
<th>Corrected integrated density (Normal*)</th>
<th>Diabetic†</th>
<th>Hypothyroid‡</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Diabetic</td>
<td>Hypothyroid</td>
<td></td>
</tr>
<tr>
<td></td>
<td>normal</td>
<td>0.64 ± 0.018</td>
<td>0.124 ± 0.0096</td>
</tr>
<tr>
<td>65</td>
<td>0.344 ± 0.047</td>
<td>0.233 ± 0.0805</td>
<td>0.461 ± 0.04</td>
</tr>
<tr>
<td>71–72</td>
<td>0.210 ± 0.081</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>97</td>
<td>0.006 ± 0.003</td>
<td>0.800 ± 0.1995</td>
<td>0.146 ± 0.0425</td>
</tr>
<tr>
<td>106</td>
<td>0.006 ± 0.003</td>
<td>0.963 ± 0.1538</td>
<td>0.004 ± 0.004</td>
</tr>
<tr>
<td>245</td>
<td>0.192 ± 0.064</td>
<td>0.004 ± 0.0025</td>
<td>0.060 ± 0.0025</td>
</tr>
<tr>
<td>246</td>
<td>0.356 ± 0.172</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>247</td>
<td>0</td>
<td>0.440 ± 0.205</td>
<td>0.337 ± 0.088</td>
</tr>
<tr>
<td>256</td>
<td>0.072 ± 0.009</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>257</td>
<td>0</td>
<td>0.237 ± 0.115</td>
<td>0.184 ± 0.022</td>
</tr>
<tr>
<td>361</td>
<td>0.434 ± 0.120</td>
<td>0.005 ± 0.0025</td>
<td>0.013 ± 0.0035</td>
</tr>
<tr>
<td>362</td>
<td>0</td>
<td>0.285 ± 0.103</td>
<td>0.383 ± 0.042</td>
</tr>
<tr>
<td>118</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>280</td>
<td>0.682 ± 0.115</td>
<td>0.629 ± 0.032</td>
<td>0.691 ± 0.120</td>
</tr>
</tbody>
</table>

Results are expressed as means ± SD. 

* n = 6; † n = 6; ‡ n = 6. 

Table 1 includes results obtained with cardiac RNA from diabetic and hypothyroid animals and the other in the hearts of hormone-deficient animals (Table 1). Although the relative predominance of each product (247, 257, and 362) appeared to vary slightly in the absence of either hormone (Table 1), these differences were within

198-fold, respectively, while those indicated by arrows in Figure 1B, as well as products 247, 257, and 362, were detected only in the fluorograms of products coded for by diabetic RNA. The relative predominance of most of these products reverted to the pattern typical of control products when RNA isolated from diabetic animals receiving daily injections of 2 U of protamine zinc insulin for 3 weeks was translated in the rabbit reticulocyte lysate. However, products 97 and 106 remained slightly elevated after insulin therapy, and neither product 361 nor product 362 was detected. The products identified by arrows in Figure 1 could not be adequately quantified and will not be further considered.

Diabetes-induced changes in the predominance of the remaining 13 specific translation products were quantified by digital matrix photometry. Since the relative predominance of nine of the 13 products was similarly affected by diabetes and hypothyroidism, the quantified determinations presented in Table 1 also include results obtained with cardiac RNA from hypothyroid animals. At least two translation products were found to be exclusively affected by the lack of insulin: product 65 was decreased 2.3-fold and product 106 was increased 198-fold. Neither of these products was significantly altered by the lack of thyroid hormone. In addition, product 97 was significantly less increased (26- vs. 141-fold) and product 245 was significantly less decreased (3.2- vs. 48-fold) by the lack of thyroid hormone than by the lack of insulin. Thus, at least four products appear to be preferentially regulated by insulin. The remaining nine products responded similarly to either insulin or thyroid hormone. For clarity, all the changes induced by insulin and thyroid hormone are presented as a composite diagram in Figure 2.

Six of the products could be identified as pairs of products having similar molecular weights but slightly different pl. They are products 246–247, 256–257, and 361–362 (Fig. 2). In each pair, one of the products was expressed in the heart of control animals and the other in the hearts of hormone-deficient animals (Table 1). Although the relative predominance of each product (247, 257, and 362) appeared to vary slightly in the absence of either hormone (Table 1), these differences were within

FIGURE 2. Diagram of insulin- and thyroid hormone-induced changes in spot predominance. The diagram summarizes the hormone-induced changes in spot predominance observed in all experiments. Decreased (Δ) or increased (O) synthesis in hypothyroid and diabetic samples. ▽ = decreased synthesis in hypothyroid samples only; ▼ = decreased synthesis in diabetic samples only; ◊ = increased synthesis in hypothyroid samples only; ◐ = increased synthesis in diabetic samples only. Other symbols as described in the legend to Figure 1.
experimental error. Products 71 and 72, which were quantified as a single product, were completely repressed in diabetic or hypothyroid animals. The repression of these products did not coincide with the coordinate appearance of a new product in hormone-deficient hearts. Finally, the abundance of RNA coding for product 41, which was increased in both hormone-deficient groups, was the only hormone-inhibited RNA product to be found in significant amount in control hearts. Two thyroid hormone-sensitive products, spots 253 and 254 (Fig. 2), were not affected by insulin.

Discussion

In the present study, we examined the diabetes-induced alterations in cardiac RNA by two-dimensional gel analysis of products translated in vitro. We used total cardiac RNA to direct proteins synthesis. Thus, products coded for by poly(A)-rich and poly(A)-minus RNA were included in this analysis, and the differences observed between control and diabetic translation products cannot be accounted for by the loss of individual RNA species which may be present as nonpolyadenylated RNA, depending on the hormonal state of the animals. A possible alteration in translational efficiency of individual RNA cannot be ruled out, however. Hormonal regulation of mRNA translational efficiency has been reported (Miles et al., 1981; Cordell et al., 1982; Mueckler et al., 1983), and it was postulated that newly synthesized mRNA may be more efficiently translated. In the present study, the animals were kept diabetic for 4 weeks and were in steady state. It is therefore unlikely that the observed decrease or increased predominance of specific translatable RNA is related to an increase or decrease of newly synthesized RNA. The possibility that hormones regulate the activity of enzymes involved in mRNA modification, such as internal methylation of adenosine residues, which may affect translational efficiency, cannot be ruled out by the present analysis. This possibility can be investigated once we have selected specific DNA clones complementary to hormone-sensitive mRNA.

Although the total RNA present in the heart of diabetic animals was significantly decreased, the ratio of translatable mRNa to total RNA measured by the amount of cardiac RNA required to saturate the modified rabbit reticulocyte lysate system remained constant. Thus, ribosomal RNA and total mRNA appear to be decreased to the same extent by diabetes. Similar results were obtained when cardiac RNA was prepared from hypothyroid animals (Dillmann et al., 1983). It is possible that decreased thyroid hormone levels observed in diabetic rats (Chopra et al., 1981; Las et al., 1981; Vadlamudi et al., 1983) may be responsible for the decreased cardiac RNA content in this group of animals. This possibility appears unlikely, since at least two translation products, spots 253 and 254, affected by the lack of thyroid hormone (Dillmann et al., 1983) are present at control levels in the diabetic heart. It is more likely, therefore, that the observed decrease in total RNA content results directly or indirectly from altered insulin levels.

Comparison of cellular translatable mRNA by two-dimensional analysis of their products translated in vitro presents several limitations. Some of these limitations result from the quantification of translated peptides by digital matrix photometry. It should be pointed out that, although spot migrations are highly reproducible, as shown by the small standard deviations obtained from pi and molecular weight measurements (Dillmann et al., 1983), the quantification of each spot is heavily dependent on the quality of the fluorograms. Since computer analysis corrects for background surrounding the measured spots, spots surrounded by a dark background may be underestimated. The variation from gel to gel is reflected by the large standard deviations obtained with spot 245-362 which far exceed expected biological variations. It is evident that translational products can be quantified with only limited accuracy, and that the hormonal influence on the relative abundance of compared products can be established with confidence only for those spots exhibiting statistically significant differences in spite of their large standard deviations. In addition, the method is severely lacking in sensitivity, so that less than 10% of the total putatively expressed proteins can be detected (Ivarie et al., 1981; Dillmann et al., 1983; Liaw et al., 1983). A total of 430 peptides could be routinely visualized by fluorography, slightly more than the number of peptides visualized by silver staining of total cardiac proteins (Dillmann et al., 1983), but significantly less than the number of peptides synthesized in isolated myocytes (Dillmann et al., unpublished observation). The number of peptides synthesized by cardiac RNA in the modified reticulocyte lysate is similar to or greater than that reported for mouse cardiac RNA (Ouellette et al., 1983), mouse liver, kidney, or brain RNA (Anzai et al., 1983), rat liver or kidney RNA (Liaw et al., 1983), or rat small intestine RNA (Kessler and DeLuca, 1985), and may indicate that a large number of exogenous RNA species cannot be efficiently translated. This becomes apparent when we compare the relative abundance of the myosin light chains to that of actin and myosin heavy chain; the former are much more heavily labeled than the latter, in spite of the greater mass of actin and myosin heavy chain RNA present in the heart. Thus, the present analysis is restricted to the very abundant RNA and those moderately abundant RNA which are efficiently translated in the reticulocyte lysate. Because of these limitations, it is also not possible to determine whether the individual mRNA not observed under specific conditions are completely repressed or simply expressed at levels not detectable by this method. Most of the RNA falling into this category (products 71-72, 246-247, 256-
257, and 361–362) are expressed at low levels, even in the induced state. Finally, the majority of translation products do not comigrate with cardiac proteins synthesized in vivo (Dillmann et al., 1983) and become difficult to identify with cellular proteins of known function.

Nevertheless, this method is adequate to analyze biochemical alterations induced during hypothyroidism and diabetes, and provides a framework for the isolation of complementary deoxyribonucleic acid (cDNA) clones which can subsequently be used to quantify hormone-sensitive specific mRNA species. A total of 19 translation products are consistently affected by diabetes. Among the 13 products which could be adequately quantified, four products were preferentially altered in the diabetic state, but only two of these products (65 and 106) were exclusively influenced by insulin. Products 97 and 106 remained slightly elevated after insulin therapy, indicating that daily injections of 2 U of insulin are not adequate to correct completely the diabetic state of these animals. Alternatively, streptozotocin, which is known to induce insulin resistance in newborn rats (Trent et al., 1984), may do so in all rats regardless of age. Indeed, phosphorylation of the insulin receptor, which is thought to be involved in cellular response to insulin, is decreased in streptozotocin-diabetic rats, and this abnormality is only partially corrected by insulin therapy (Kadowaki et al., 1984). Since not all insulin-sensitive cellular parameters are equally sensitive to insulin, it is possible that limited insulin resistance which may develop in streptozotocin-diabetic animals is responsible for the diminished response of spot 106 and 97 to insulin treatment. Comparison of data obtained in this study to those obtained previously (Dillmann et al., 1983) shows that two other products (253 and 254) are exclusively affected by hypothyroidism. The remaining nine products, two of which (spot 41 and 97) were not quantified in the previous report (Dillmann et al., 1983), are similarly regulated by thyroid hormone and insulin. Six of the hormone-responsive products are pairs of peptides of similar molecular weight and slightly different pl. Since these products were synthesized in vitro, they cannot represent posttranslational modifications of the same product, and although they have not been identified yet, they may represent isoforms of the same peptide. Only one putative isoform would be expressed under a given hormonal state. Such coordinate expression of two isoforms of the same product by thyroid hormone and insulin has been reported for at least one identified cardiac protein, the myosin heavy chain (Dillmann et al., 1984). In addition to a common set of products affected by insulin or thyroid hormone, each hormone appears to affect specifically a small number of products not affected by the other hormone.

The mechanisms by which thyroid hormone and insulin affect translatable mRNA levels in the heart remain unknown. Decreases in thyroid hormone levels occur in diabetic rats (Chopra et al., 1981; Las et al., 1981; Vadlamudi et al., 1983). However, it appears unlikely that the overlap in the response of specific peptides results from the lowered thyroid hormone levels, because not all thyroid hormone-responsive products are affected in the diabetic heart. In a previous study (Dillmann, 1982), it was found that treatment of diabetic rats with physiological doses of T3 had no influence on the altered myosin isoenzyme distribution which occurs in both diabetic and hypothyroid hearts. Similarly, not all insulin-responsive translation products are changed in hypothyroid hearts, even though insulin levels are decreased in hypothyroid rats (Aranda et al., 1972). Therefore, it is more likely that these cardiac peptides are under multiple hormonal regulation, as has been established for most mRNA expressed in other mammalian tissues. Whether changes in the cardiac translation products, which respond in a similar fashion to thyroid hormone or insulin, are direct effects of each hormone via receptors occupancy, or whether they are caused by similar secondary effects generated independently by the two hormones, remains to be elucidated.

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