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Effect of Physiological Concentrations of Insulin and Antidiabetic Drugs on RNA Release From Isolated Liver Nuclei

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The addition of 10^{-11}M insulin to a cell-free system from rat liver promotes the release of messengerlike RNA from isolated prelabeled nuclei. The stimulation was similar whether the nuclei were preincubated with insulin, or if insulin was added directly to the cell-free system with or without a protease inhibitor. Dot blot hybridization using cloned cDNA for α_{2u}-globulin mRNA showed that this was one of the messages whose release was enhanced by insulin. Nuclei isolated from rats treated with either of the antidiabetics tolbutamide or tolazamide showed no increase in RNA release in the presence of insulin over the concentration range 10^{-5}–10^{-14} M. Furthermore, these nuclei did not release detectable levels of α_{2u}-globulin mRNA.

Key words: insulin, anti-diabetic drugs, tolazamide, tolbutamide, RNA transport, α_{2u}-globulin mRNA

Insulin is known to produce both immediate and late metabolic effects. The latter may be associated with the internalization of the hormone and its binding to high-affinity sites on the nuclear membrane [1,2]. Insulin binding to the nuclear membrane has been shown to stimulate the nuclear pore ATPase [3] and mRNA, but not rRNA, efflux from the nucleus to the cytoplasm in a cell-free system [4]. Since these latter experiments required insulin concentrations greater than physiological, it was of interest to determine the cause of the high insulin requirement and, if possible, to modify the system to respond to lower insulin levels.

Among the specific long-term effects of insulin is the enhancement of the concentration of mRNA for albumin [5], α_{2u}-globulin [6], and tyrosine amino transferase [7]. If nuclei incubated in the cell-free system, which has previously been shown to release functional mRNA [8], are responding to insulin as do nuclei in vivo, it should be possible to detect an increased amount of these mRNAs in the RNAs released in vitro in the presence of insulin. The availability of a cloned cDNA to α_{2u}-globulin mRNA [9] makes such a study feasible at this time.

One of the acute in vivo effects of the oral antidiabetic drugs tolbutamide and tolazamide is to increase the levels of circulating insulin. These drugs have been

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reported to increase cellular insulin binding in insulin-independent diabetics (type II) by increasing the number of insulin receptors on target cells [10]. Whether there is an increase in nuclear binding of insulin or other postreceptor effects is unknown. Since nuclei incubated in the cell-free system respond to insulin, this system is ideal for testing the nuclear effects of these drugs.

METHODS

Animals employed for these studies were male Sprague-Dawley rats weighing 200–250 g. Rats were fasted overnight and then injected with 100 μCi of ^{3}H-orotic acid (i.p.) 30 min before livers were removed and nuclei prepared [11]. Cytosol was prepared from fasted or unfasted rats as described previously [12]. The cell-free system consisted of 5×10^6 nuclei/ml incubated in medium containing 50 mM Tris-HCl (pH 7.5), 25 mM KCl, 2.5 mM $MgCl_2$, 0.5 mM $CaCl_2$, 0.3 mM $MnCl_2$, 2.5 mM Na_2HPO_4, 5.0 mM NaCl, 5.0 mM spermidine, 2.0 mM dithiothreitol, 2.0 mM ATP, 2.5 mM phosphoenol pyruvate, 35 units/ml of pyruvate kinase, 300 μg/ml yeast RNA, 0.15 mM aurintricarboxylic acid, and 12 mg/ml cytosol protein. Insulin at 10^{-5}–10^{-14} M was added directly to the cell-free system with or without the addition of 0.1 units/ml of aprotinin as a protease inhibitor. In some experiments, before addition to the cell-free system, nuclei were preincubated for 10 min at 0°C in the presence of the indicated concentration of insulin in a buffer containing 50 mM Tris-HCl (pH 7.5), 5.0 mM $MgCl_2$, 25 mM KCl, 5.0 mM spermidine, 0.5 mM $CaCl_2$, 0.3 mM $MnCl_2$, and 0.25 M sucrose [13]. All incubations of the cell-free system were at 30°C for the indicated time. Nuclei were removed by centrifugation, and the supernatant protein was precipitated with 5% trichloroacetic acid. The precipitate was washed with ethanol, dissolved, and counted in liquid scintillant.

For experiments involving the antidiabetic drugs tolbutamide and tolazamide (Upjohn, Kalazamoo, MI), the drugs were injected i.p at 50 mg/kg and 8 mg/kg, respectively. For cytosol, the livers were removed 4 hr after drug treatment for tolbutamide and 1 hr for tolazamide. For nuclei, 100 μCi of ^{3}H-orotic acid was injected 3.5 hr after drug treatment for tolbutamide and 45 min for tolazamide. In both cases, the liver was removed and nuclei prepared 30 min after the isotope was injected.

For dot blot hybridizations, the labeled mRNA was purified from the postnuclear supernatant by phenol extraction at pH9 [14], precipitated with ethanol and reprecipitated in the presence of 4.0 M guanidine hydrochloride [15]. Recombinant plasmid DNA, containing the insert (Pα176) for rat α_{2u}-globulin cDNA [9,16] was amplified in Escherichia coli MM 294 [17] and extracted by a cleared lysate procedure [18]. Recombinant DNA was purified by banding in cesium chloride-ethidium bromide density gradients. The DNA was dialyzed, concentrated by ethanol precipitation, restricted with Sal I, phenol-extracted, and reprecipitated with ethanol. It was then 5′-end-labeled [19] using prepared reagents (BRL, Bethesda, Md). RNA was bound to nitrocellulose paper [20] and hybridized with the end-labeled DNA using the procedure of Lacker [21].

RESULTS

In a previous publication, we demonstrated that the addition of 3×10^{-7}M insulin to a cell-free RNA transport system containing cytosol from fasted rats caused

an increase of approximately 50% in the release of messengerlike RNA from prelabeled rat liver nuclei. The optimal insulin concentration could be decreased by lowering the cytosol concentration or increased by raising it. This suggested that cytosol proteins were interfering with the nuclear insulin effect. To minimize the binding of added insulin to any cytoplasmic proteins, it was decided to alter the standard cell-free system by using cytosol prepared from unfasted rats. Cytosol isolated from these rats would be expected to contain a higher concentration of internalized insulin than cytosol from fasted animals. Consequently, any insulin-binding sites in the cytosol would be expected to be filled. In addition, the presence of internalized insulin in unfasted rat cytosol should inhibit intracellular proteolysis [22].

As shown in Figure 1, there was a stimulation of RNA release when nuclei were incubated with insulin in the cell-free system containing cytosol from nonfasted animals. In contrast to the results reported earlier using cytosol from fasted animals [4], two peaks of insulin stimulation were observed. These peaks occured at 10^{-7} M, the same as the single optimum seen with cytosol from fasted animals, and 10^{-11} M, the latter being within the physiological range. The addition of aprotinin as a protease inhibitor did not alter either of the optimal insulin concentrations. This suggested that the previously observed cytosol interference was probably not due to degradation of insulin by proteases, but this has not yet been tested directly. To further test this possibility, nuclei were preincubated in the absence of cytosol for 10 min at 0°C with various concentrations of insulin using the method of Goldfine [23]. These nuclei were then added to the cell-free system and incubated at 30°C for 30 min. As can be seen in Figure 1, this procedure gave the same insulin optima as did the direct addition

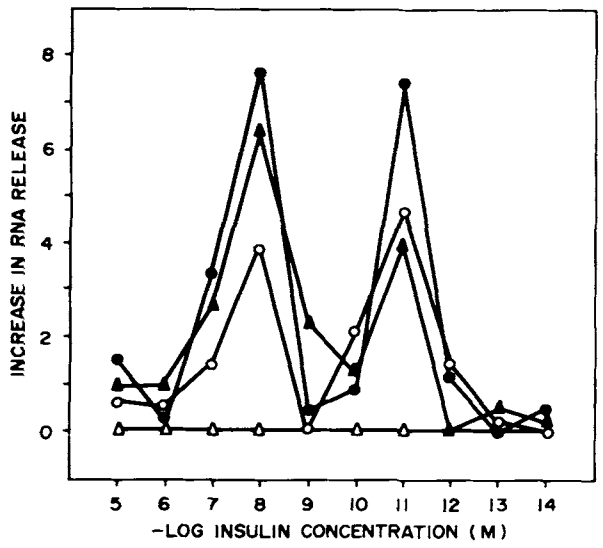

Fig. 1. Effect of various insulin concentrations on release of RNA from prelabeled nuclei to a cell-free system containing cytosol from unfasted animals. The ordinate is % of nuclear counts released in the presence of insulin less the % released in its absence. Preincubation of nuclei with insulin for 10 min at 0°C prior to the addition of the other components of the cell-free system. ●—●, direct addition of insulin to the complete cell-free system; ○—○, direct addition of insulin to the cell-free system to which 0.1 units of aprotinin had been added; ▲—▲, direct addition of insulin to cell-free system containing nuclei from a 60-min tolazamide-treated rat △—△.

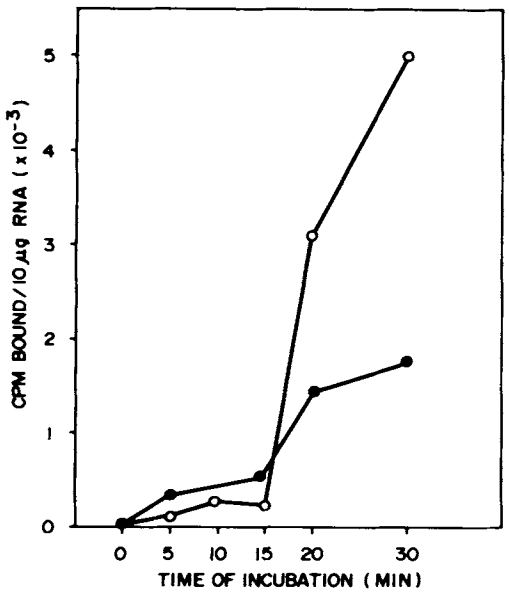

Fig. 2. Dot blot hybridization of 5′-end-labeled cDNA to α_{2u}-globulin mRNA to RNA released from isolated nuclei in the presence (○—○) and absence (●——●) of 10^{-11} M insulin.

of insulin to the complete cell-free system. Furthermore, preincubation gave the same results whether fasted or unfasted rat cytosol was used.

Insulin is known to enhance the hepatic concentration of the mRNA coding for α_{2u}-globulin [6]. Using 5′-end-labeled cloned cDNA for α_{2u}-globulin mRNA and dot blot hybridization, we tested whether the release of α_{2u}-globulin was enhanced in the presence of 10^{-11} M insulin. The results of this experiment are shown in Figure 2. While there was little release of α_{2u}-globulin mRNA in either system during the first 15 min of incubation, the enhancement of release of this mRNA by insulin in the latter time points is clearly observed. Thus the effect of physiological concentrations of insulin in the cell-free system is similar to that found in vivo in regard to enhancing the accumulation of α_{2u}-globulin mRNA.

In order to test whether the antidiabetic drugs tolbutamide and tolazaminde, would sensitize nuclei to added insulin, rats were injected with these drugs and 30-min prelabeled nuclei prepared at the time of peak drug effectiveness (see Methods). At insulin concentrations from 10^{-5} to 10^{-14} M, there was no increase in RNA release when these nuclei were incubated in the cell-free system (Fig. 1). The nonresponsiveness was not altered by the substitution of cytosol from normal as opposed to drug-treated rats.

In spite of the lack of response of drug-treated nuclei to in vitro insulin, there was the possibility that, due to the drug-induced increase in circulating insulin, such nuclei might still release increased amounts of α_{2u}-globulin mRNA. However, dot blot hybridization using an α_{2u}-globulin cDNA probe showed no evidence of α_{2u}-globulin mRNA in the RNAs released from tolazamide treated nuclei (results not shown).

DISCUSSION

In eukaryotic cells, there exist multiple sites for the regulation of genetic expression. Control at the levels of transcription and translation have been extensively studied. Control at the levels of RNA processing and nucleocytoplasmic transport have only recently begun to be investigated. We have developed a cell-free system for RNA processing and transport which has been shown to release functional messenger [8] and ribosomal RNA [24]. The release requires a temperature greater than 15°C, a hydrolyzable nucleoside triphosphate, and particular soluble cytosol proteins [12]. We have previously shown that the release of messenger RNA in this cell-free system can be modulated by physiological levels of cAMP and cGMP [25]. We have also shown a significant increase in the release of messenger, but not ribosomal, RNA in the presence of insulin but only at insulin concentrations above the physiological level. Analysis of the nuclear RNA indicated that the insulin effect was due primarily to stimulation of RNA release rather than processing.

If the cell-free system is modified to utilize cytosol from a fed rather than a fasted rat, two insulin optima are observed. One is at 10^{-7} M, the same as with fasted cytosol; the other, at 10^{-11} M. The former may be due to cross-reaction of insulin with a nuclear receptor for somatomedin or an insulinlike growth factor. Because of its low K_m, the 10^{-11} M optima receptor probably represents a genuine insulin receptor. Specific binding of insulin to nuclei was reported by Goldfine in 1977 [1] and has since been confirmed by several other laboratories [2,26]. The structure of the nuclear receptor has been recently published [27]. The K_m for nuclear insulin binding is similar to the concentration of insulin that produces a fourfold increase in the release of the messenger RNA for α_{2u}-globulin. Since overall RNA release at this insulin concentration increases only 50%, the effect on α_{2u}-globulin appears to be specific. However studies of other insulin responsive mRNAs will be necessary to confirm this specificity. Our previous work indicated that the major effect of insulin was to increase RNA release with little effect on processing [4]. However, the kinetics of release of α_{2u}-globulin mRNA suggest that for this mRNA, insulin may also effect processing. In the absence of insulin, much of the previously synthesized α_{2u}-globulin mRNA would remain unprocessed in the nuclei, while in its presence, the mRNA would be processed and released. Experiments employing intron-specific probes are currently underway to test this hypothesis.

Since the cell-free system appears to show insulin responses similar to those observed in vivo, it will be of interest to use this system to explore the nuclear response to insulin in diabetic rats. We have already shown [4] that nuclei from the obese Zucker rat, a model for adult onset diabetes, do not respond to insulin in the cell-free system. Their ability to transport insulin-responsive mRNAs has not yet been tested. Purcello et al [28] have examined the insulin response of nuclei in streptozotocin-diabetic rats and found an increase in RNA release, a stimulation of the nuclear pore ATPase, and a decrease in ^{32}P incorporation at 10^{-11} M insulin. The effect of insulin on specific mRNAs in streptozotocin-diabetic rat nuclei has not yet been examined.

Acute treatment by the antidiabetic drugs tolazamide and tolbutamide is known to increase the level of circulating insulin. On longer treatment, these drugs have also been reported to increase the number of insulin receptors in the plasma membrane [10] or to alter only postreceptor actions of insulin [29,30]. The effect of these drugs

on the nuclear receptor is unknown. Nuclei of tolazamide-treated rats incorporate approximately the same amount of labeled orotic acid as do the nuclei of untreated rats. In the absence of insulin, the overall release of RNA is only slightly higher than with untreated rats. This would suggest that the drug-treated animals do not have a higher level of newly synthesized HnRNA, nor do they process and transport mRNA at a significantly greater rate than do controls. However, there is no increase in RNA release in the presence of exogenous insulin when nuclei have been pretreated in vivo with either tolazamide or tolbutamide. This suggests that either all the nuclear insulin receptors are occupied or the number of receptors has been drastically reduced. Saturated receptors would be expected to stimulate the release of insulin responsive mRNAs such as that for α_{2u}-globulin. This does not occur. Thus, it appears that under acute treatment at times of peak drug effectiveness, the number of nuclear receptors is reduced. A direct measurement of the number of receptors will be required to confirm this hypothesis. Further, a study of insulin binding and α_{2u}-globulin mRNA processing and release at times prior to peak drug effectiveness and on chronic treatment would help to elucidate the mechanism of action of these drugs.

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