INSULIN-MEDIATED INHIBITION OF TYROSINASE ACTIVITY
AND PROTEIN SYNTHESIS IN MELANOMA CELL CULTURES

by

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ABSTRACT

Insulin lowers basal levels of tyrosinase activity and inhibits the MSH-stimulated rise in tyrosinase in Cloudman S-91 mouse melanoma cell cultures. These cultures are very sensitive to insulin. A concentration of insulin as low as 5×10^{-7} M insulin produces optimum inhibition. At maximum inhibition, tyrosinase activity is reduced to approximately 50% of control levels. Insulin inhibits cellular proliferation in melanoma cells; however, inhibition of tyrosinase activity precedes this effect. Insulin also inhibits the $(\text{Bu})_2$ cAMP and theophylline stimulated rise in enzyme activity. This finding suggests that insulin exerts its effects at a site distal to cAMP production. Insulin, in fact, does not lower cAMP levels in melanoma cells, nor does it alter the MSH-stimulated rise of cAMP. The inhibitory effect of insulin on tyrosinase activity could not be mimicked by either $(\text{Bu})_2$ cGMP or 8-bromo-cGMP, suggesting that insulin does not exert its effects by altering cellular levels of this nucleotide. Insulin decreases the incorporation of $[3\text{H}]$ -leucine into trichloroacetic acid insoluble material by 50%, an inhibition which corresponds well with the observed level of reduction of tyrosinase activity. This finding suggests that the inhibition of tyrosinase activity may be caused by a general reduction in protein synthesis due to insulin treatment.

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CHAPTER I

INTRODUCTION

Insulin is a polypeptide hormone consisting of two peptide chains linked by a disulfide bond. It is synthesized from a single polypeptide chain precursor called proinsulin, which is cleaved to form the mature insulin molecule. Insulin is secreted into the circulation by the B-cells of the pancreas and its principle targets are the liver, muscle, and adipose tissues. It plays a major role in the regulation of carbohydrate, amino acid, and fat metabolism. It facilitates the utilization of glucose and promotes the synthesis of glycogen, protein, and lipids.

Numerous diverse effects of insulin on cellular metabolism have been observed, and evidence suggests that these effects are triggered by its interaction with cell surface protein receptors. However, a single unique action of insulin leading to all the various intracellular phenomena resulting from the hormone has not been determined. Generalized increases in protein (1,2), RNA (3), and DNA (4) synthesis in cells exposed to insulin have been reported. Studies have shown that insulin can increase mRNA levels for specific proteins including albumin (5), alpha-amylase (6), and tyrosine aminotransferase (7). In these studies, rats were rendered diabetic with streptozotocin (6) or alloxan (5), which reduced mRNA levels of the respective proteins. Treatment with insulin restored mRNA levels to normal.

In contrast to most reports describing stimulatory effects of insulin on cellular metabolic processes, PEP carboxykinase (PEPCK) mRNA levels have been shown to decrease following insulin treatment (8). In this study, the specificity of the effect of insulin and the interaction of insulin with its specific receptor was reported by comparing the actions of insulin and proinsulin. Insulin binds receptor 15-20% more avidly than proinsulin, and in this study a 1.4 log difference between the two for binding and repression of PEPCK mRNA correlates very well with this data. This supports a current model of insulin action which implies that interaction with insulin receptor at the cell surface initiates the subsequent effects of the hormone. Another study supporting this notion is reported by Cuatrecasas (9). He showed that insulin covalently linked to Sepharose polymers was almost as effective as native insulin in inhibiting lipolysis and stimulating glucose transport in rat fat cells. Since the insulin derivatives cannot enter the cells, this indicates that interaction of insulin with cell surface structures initiates a variety of metabolic alterations. Other reports have suggested that the entry of insulin or one of its degradation products into the cell mediates the intracellular functions of insulin.

Kahn and Baird (10) have shown that insulin bound to adipocytes undergoes rapid internalization, compartmentalization, and degradation into higher and lower MW compounds. By exposing adipocytes to ^{125}I insulin and treating them at intervals with a variety of dissociating agents, such as trypsin, the bound insulin became increasingly less dissociable, suggesting that bound insulin is transferred inside the

cell where it is less accessible to the dissociating agents. Also, specific binding sites for insulin have been found on a variety of intracellular structures including Golgi membranes (11) and nuclei (12). It is also possible that insulin generates an unknown "second messenger" through its interaction with the cell membrane, which relays signals into the cells' interior.

Few studies have been directed at examining insulin effects on melanoma cells in culture. Insulin has been shown, in one study, to arrest proliferation of Cloudman S-91 mouse melanoma cells at a concentration of 10^{-8}M (13). This was thought to be due to a lowering of cAMP levels, an effect of insulin which has been reported in other cell types. However, Mather and Sato (14) have demonstrated that insulin is a requirement of mouse melanoma cell growth and they have developed an insulin containing defined medium for the growth of melanoma cells. They also showed that insulin increases the incorporation of glucose into glycogen and fatty acids, thereby demonstrating that melanoma cells respond to insulin in the same manner as target cells such as fat and liver. It has recently been shown that Cloudman S-91 melanoma cells have relatively high levels of insulin binding and that insulin binds to surface receptors throughout the cell cycle with the highest binding occurring during the S phase (15). This finding raises the possibility of regulation of cellular response to insulin by receptors which are functional at particular phases of the cell cycle. It was also observed that insulin undergoes internalization and degradation 30 minutes after binding to surface receptors (11).

No studies have been carried out to investigate the effects of insulin on differentiation events in mouse melanoma cell cultures. The present studies were undertaken to examine the effects of insulin on the activity of tyrosinase, the rate limiting enzyme for melanin synthesis. Mouse melanoma cells respond to the peptide hormone MSH by demonstrating increased activity of tyrosinase (16). The results presented here reveal that insulin not only reduces the basal level of tyrosinase activity, but inhibits the MSH-induced stimulation of the enzyme as well. The studies performed involved examination of insulin on tyrosinase activity, the effects of different concentrations of insulin on basal tyrosinase levels and upon MSH-stimulated tyrosinase activity, the effects of insulin on tyrosinase activity as measured in cell homogenates, effects of insulin on cAMP levels in melanoma cells, effects of insulin on the theophylline and dibutyryl cAMP stimulation of tyrosinase, effects of cGMP analogues on tyrosinase activity in melanoma cells, and the effect of insulin on transcription and translation in melanoma cell cultures.

CHAPTER II

MATERIALS AND METHODS

Melanocyte Stimulating Hormone (alpha-MSH), insulin, N,O'-di-butyryl cyclic AMP [(Bu)₂cAMP], theophylline, 8-Bromocyclic GMP (8-Br-cGMP), and N,O'-dibutyryl cyclic GMP [(Bu)₂cGMP] were purchased from Sigma Chemical Co. (St. Louis, MO). Ham's nutrient mixture F-10 was obtained from Grand Island Biological Co. (Grand Island, N.Y.). Horse serum was purchased from Sterile Systems, Inc. (Logan, Utah). Radioisotopes including [ring-e,5-³H]-tyrosine (sp. act. 50 Ci/mmole), and L-[4,5-³H(n)] -leucine (60 Ci/mmole) were purchased from New England Nuclear (Boston, MA).

Cell Cultures

Cloudman S-91 (CCL 53.1) mouse melanoma cells were obtained from the American Type Culture Collection Cell Repository. Cells were grown in Ham's F-10 medium fortified with 10% horse serum. Penicillin/streptomycin (100 units/ml and 100 ug/ml, respectively) were also present in the medium. Stock cultures were subcultured weekly. Cell counts were made using a hemacytometer.

Tyrosinase Assays

To determine the effect of insulin or other compounds on tyrosinase activity in cell cultures, cells were seeded at the densities indicated and left overnight. At the start of experiments the

medium was removed and replaced with fresh medium containing the compounds under investigation. For 24 hour experiments, the medium also contained 1 uCi/ml of [^3H]-tyrosine. Following a 24 hour exposure, medium was removed and assayed for the presence of $^3\text{H}_2\text{O}$. For 48 hour experiments, no [^3H]-tyrosine media was used for the first 24 hour exposure period to hormones. At the end of the first 24 hour incubation period, medium was removed and replaced with medium containing 1 uCi/ml of [^3H]-tyrosine and the hormone or compound being studied. At the end of this second 24 hour exposure, medium was removed and assayed for $^3\text{H}_2\text{O}$. The amount of $^3\text{H}_2\text{O}$ formed was determined by using a modification of the charcoal method of Pomerantz (17,18). Following absorption of the [^3H]-tyrosine by charcoal, the liquid phase was passed over a Dowex 50W column as described elsewhere (19).

To determine tyrosinase activity in cell homogenates, cell cultures were exposed to various hormones for the times indicated. At the end of this exposure period cells were removed from flasks and homogenized in 20 mM sodium phosphate buffer (pH 7.0) containing 0.1% Triton X-100. Homogenates were incubated with 1 umole/ml L-tyrosine, 0.1 umole/ml L-3,4-dihydroxyphenylalanine, 3 uCi/ml [^3H]-tyrosine and 50 mM sodium phosphate (pH 6.8) in a total volume of 0.5 ml at 37°C for 4 hours. The amount of $^3\text{H}_2\text{O}$ formed during this time was determined as described above.

Protein Synthesis Measurements

The effect of insulin on protein synthesis was determined by measuring the incorporation of [^3H]-leucine into TCA-precipitable

material as previously described (20). Briefly, cells, seeded into 25 cm² flasks (2 x 10⁵ cells/flask), were exposed to insulin for the times indicated. Following this incubation period cells were pulsed with 8 uCi/ml of [³H]-leucine in Ham's F-10 medium for 60 minutes. Flasks were then washed three times with Hank's Balanced Salt Solution (HBSS), 3 ml of 10% TCA was added, and the flasks left overnight at 4°C. The TCA was then removed and saved and the flasks were washed twice in 5% TCA and twice in ethanol. Three ml of 1 N NaOH were then added to the flasks and the protein extracted by incubation at 37°C for 6 hours. Samples were made acidic (pH 6.0) with 1 N HCl, scintillant was added, and the samples counted. To determine cellular uptake of label, the 10% TCA supernatant fraction was counted.

Determination of cAMP Levels

Intracellular cAMP levels were measured by the use of a protein binding assay kit for cAMP purchased from Amersham Corp. (Arlington Heights, Ill.). Cells were seeded (5 x 10⁵ cells/flask) into 25 cm² culture flasks and allowed to attach overnight. Flasks were then exposed to hormones (or left untreated) and at the times indicated, medium was removed, the flasks washed once in cold HBSS, and 3 ml of cold ethanol added immediately. Flasks were left at 4°C for 24 hours after which time the ethanol fraction was removed and evaporated under N₂. The dried residue was resuspended in 0.05 M Tris buffer (pH 7.5) containing 4mM EDTA. Following centrifugation the supernatant was assayed for cAMP.

CHAPTER III

RESULTS

Previous reports have shown that insulin inhibits proliferation in Cloudman melanoma cells (13), and we performed our initial experiments to confirm this effect of insulin. Significant inhibition of growth by 10^{-7} M insulin was observed only after cells had been treated with the hormone for 3 days (Fig. 1). We next investigated the effects of insulin on tyrosinase activity. Although some inhibition on enzyme activity was apparent after a 24 hour exposure of cells to insulin (15% inhibition), a 48 hour exposure period resulted in a reduction of basal tyrosinase levels by at least 40% (Fig. 2). Since mouse melanoma cells respond to MSH by demonstrating increased tyrosinase activity, we next investigated the dose response of insulin inhibition on basal tyrosinase levels and on the MSH-mediated increase in tyrosinase activity. Insulin at both 10^{-7} M and 10^{-8} M markedly reduced both basal and MSH stimulated enzyme levels (Fig. 2); however, insulin at 10^{-9} M neither inhibited basal levels nor suppressed the induction by MSH. Insulin inhibited induced enzyme levels from cells exposed to either 10^{-7} M or 10^{-8} M MSH to a similar degree and at both of these concentrations of MSH, insulin, at 10^{-7} M inhibited the MSH stimulated increase in enzyme activity by more than 50%. It was possible that the observed inhibitory effect of insulin on tyrosinase activity might be due to an interference by insulin with the uptake of [3 H]-tyrosine by cell cultures. The resultant

Figure 1. Insulin Effects on Cell Growth

Melanoma cells (2×10^5 cells/flask) were seeded into 25 cm² culture flasks and allowed to attach overnight. Cells were then cultured in the absence ($\bullet\text{---}\bullet$) or presence ($\square\text{---}\square$) of insulin (10^{-7}M) for 5 days. Medium (with or without insulin) was changed daily. Cell counts were determined from triplicate flasks each day for 5 days. Values are averages $\pm$ s.e.

Figure 1. Insulin Effects on Cell Growth

Figure 2. Dose Response of Insulin Inhibition of MSH Stimulated Tyrosinase Activity.

Cells were seeded at 2×10^5 cells/flask and exposed to insulin at the above concentrations and either no MSH (○—○), MSH (10^{-7} M) (■—■), or MSH (10^{-8} M) (▲—▲) for 48 hours. The medium during the second 24 hour period contained 1 uCi/ml of [3 H]-tyrosine. The amount of $^3\text{H}_2\text{O}$ produced during this time was determined as outlined in Materials and Methods. Each value represents an average of six determinations $\pm$ s.e.

Figure 2. Dose Response of Insulin Inhibition of MSH Stimulated Tyrosinase Activity.

inability of [^3H]-tyrosine to cross the cell membrane would then produce the apparent inhibitory effect on enzyme activity when no alteration of enzyme levels actually occurred. To examine this possibility, cells were exposed to insulin for 48 hours and then homogenized. The homogenates were then assayed for tyrosinase activity as shown in Table 1. The inhibition of tyrosinase activity by insulin was still evident. Therefore, the inability of [^3H] tyrosine to cross the cell membrane was not the reason for the observed inhibition of tyrosinase activity due to insulin since the membranes are eliminated by homogenization.

Several studies have demonstrated that MSH exerts its effect on melanoma cells through the second messenger cAMP. Fuller and Viskochil (21) have shown that exposure of mouse melanoma cells in culture to MSH (10^{-7} M) results in an elevation of intracellular cAMP levels which is ten times that of control levels within 20 minutes after exposure to MSH. Theophylline and the cAMP analogue, dibutyryl cAMP, can also mimic this MSH action (16,21). Therefore, it is possible that insulin may exert its inhibitory effects by either lowering intracellular levels of cAMP or by preventing the rise in cAMP levels due to MSH treatment. As shown in Table 2, insulin at 10^{-7} does not reduce basal levels of cAMP although insulin at 10^{-9} M appeared to cause a slight reduction in these levels. Insulin does not prevent the MSH-induced increase in intracellular cAMP levels at either concentration. To further explore the effect of insulin on the induction of tyrosinase through cAMP, we exposed cells to either theophylline (1mM) or dibutyryl cAMP (10^{-4} M) to determine whether or not insulin could antagonize the induction of tyrosinase caused by either of these compounds. Table 3

TABLE 1 Determination of the Effects of Insulin on Tyrosinase Activity as Measured in Cell Homogenates

Treatment	Tyrosinase Activity nmoles/mg/4 hrs	% of control
control	0.80 $\pm$ 0.10	100
insulin (10^{-7} M)	0.64 $\pm$ 0.06	79
MSH (10^{-7} M)	6.90 $\pm$ 0.31	863
MSH + insulin	2.62 $\pm$ 0.01	328

Cells were seeded at 2×10^5 cells/flask and exposed to the above hormones for 48 hours. Cell homogenates were prepared and assayed for tyrosinase activity as described in Materials and Methods. Values are the averages of four determinations $\pm$ s.e.

TABLE 2 Effect of Insulin on cAMP Levels in Melanoma Cell Cultures

Additions	Cyclic AMP pmoles/10 ⁶ cells
control	5.4 ± 0.8
MSH (10 ⁻⁷ M)	13.5 ± 1.1
insulin (10 ⁻⁷ M)	5.8 ± 0.6
insulin (10 ⁻⁹ M)	4.8 ± 1.0
MSH (10 ⁻⁷ M) + insulin (10 ⁻⁷ M)	13.5 ± 0.8
MSH (10 ⁻⁷ M) + insulin (10 ⁻⁹ M)	14.4 ± 1.6

Melanoma cells were seeded (10⁶ cells/flask) into 25 cm² culture flasks and allowed to attach overnight. Cells were then exposed to the above hormones for 30 minutes, after which time they were prepared for cAMP measurements as described in Materials and Methods. Results are the averages of four determinations ± s.d. This experiment was repeated 3 times with similar results.

TABLE 3 Effect of Insulin on the Theophylline and (Bu)₂cAMP Stimulation of Tyrosinase

Treatment	Tyrosinase Activity nmoles/10 ⁶ cells/24 hrs
control	0.19 ± 0.01
insulin (10 ⁻⁷ M)	0.09 ± 0.01
(Bu) ₂ cAMP (10 ⁻⁴ M)	0.62 ± 0.02
(Bu) ₂ cAMP (10 ⁻⁴ M) + insulin (10 ⁻⁷ M)	0.22 ± 0.01
theophylline (1 mM)	1.32 ± 0.03
theophylline (1 mM) + insulin (10 ⁻⁷ M)	0.91 ± 0.01

Conditions are as described in Figure 2 and in Materials and Methods. Values are the averages of six determinations ± s.e. Assays shown were taken at 48 hours.

illustrates that insulin inhibited the increase in tyrosinase activity in cells treated with either compound. These results indicate that insulin most likely exerts its inhibitory effects at some point in the tyrosinase induction pathway distal to the production of cAMP.

Several reports have suggested that insulin may exert its effects in some cells by altering cGMP levels. (22,23) Since insulin apparently does not function through cGMP, we next investigated the possibility that insulin may act through a cAMP mediated process. If this theory were correct, then exposure of melanoma cultures to cGMP analogues such as 8-Br-cGMP or (Bu)₂ cGMP could result in an inhibition of tyrosinase activity similar to that seen with insulin. All cultures exposed to either (Bu)₂ cGMP or 8-Br-cGMP at either 10⁻⁶ M or 10⁻⁹ M caused no inhibition of the MSH stimulated tyrosinase activity, although a slight reduction in basal enzyme activity was observed with 8-Br-cGMP at 10⁻⁶ M (Table 4). From these results, it appears that an alteration in cGMP levels is not the mechanism in which insulin inhibits tyrosinase activity.

The increase in tyrosinase activity in response to MSH is completely suppressed by addition of inhibitors of RNA or protein synthesis such as Actinomycin D or cyclohexamide (21). This suggests that both transcription and translation are necessary for the MSH mediated increase in tyrosinase activity. Because of these findings, we examined the possibility that insulin might alter either of these processes. We observed a marked decrease in the incorporation of [³H]-leucine into acid insoluble material in cell cultures treated with insulin. Insulin had no apparent effect on the uptake of [³H]-leucine into cell

TABLE 4 Effect of cGMP Analogues on Tyrosinase Activity in Melanoma Cell Cultures

Additions	Tyrosinase Activity nmoles/ 10^6 cells/24 hrs
control	0.15 ± 0.02
MSH (10^{-7} M)	2.88 ± 0.08
(Bu) $_2$ cGMP (10^{-6} M)	0.23 ± 0.02
(Bu) $_2$ cGMP (10^{-9} M)	0.14 ± 0.01
MSH + (Bu) $_2$ cGMP (10^{-6} M)	2.77 ± 0.09
MSH + (Bu) $_2$ cGMP (10^{-9} M)	2.64 ± 0.06
8-Br-cGMP (10^{-6} M)	0.11 ± 0.06
8-Br-cGMP (10^{-9} M)	0.16 ± 0.06
MSH + 8-Br-cGMP (10^{-6} M)	2.85 ± 0.11
MSH + 8-Br-cGMP (10^{-9} M)	2.78 ± 0.12

Conditions as described in Figure 2 and in Materials and Methods.
Results are the averages of 6 determinations $\pm$ s.e.

cultures (Table 5). Also, the level of inhibition of protein synthesis after a 48 hour exposure of cell cultures to insulin parallels the observed level of reduction of tyrosinase activity.

TABLE 5 Uptake of [^3H]-leucine into Acid-soluble and Insoluble Materials in Melanoma Cells Treated with Insulin

Additions	TCA-soluble	<u>[^3H]-leucine (nmoles/10^6 cells/hr)</u>	
		% of control	TCA-insoluble % of control
control	0.27 $\pm$ 0.22	100	2.59 $\pm$ 0.18 100
insulin (10^{-7}M)	0.25 $\pm$ 0.01	93	1.34 $\pm$ 0.05 52

Experimental conditions are as described in Materials and Methods.
Values are the averages of four determinations $\pm$ s.e.

CHAPTER IV

DISCUSSION

The results presented here show that insulin both lowers basal levels of tyrosinase activity and suppresses the MSH-mediated increase in the activity of the enzyme. We observed that some inhibition of enzyme activity occurs at 24 hours (15-25% inhibition) with greatly increased inhibition at 48 hours (40-50% inhibition). Our initial study demonstrated that inhibition of cell proliferation by insulin does not occur until at least day 3. Inhibition of tyrosinase activity by insulin occurs before this inhibition of cell growth as shown in Figure 1; therefore, a reduction in enzyme activity due to insulin treatment is not caused simply by a reduction in cell growth. Pawelek and co-workers (13) have demonstrated an inhibition of proliferation in Cloudman S-91 melanoma cell cultures with concentrations of insulin ranging from 10^{-10} to 10^{-8} M, while our results were observed using 10^{-7} M insulin. In contrast to these findings, Sato and co-workers have shown that insulin is required for growth of mouse melanoma and other cell lines maintained in serum free medium (14,24). The concentrations of insulin used in these studies, varying from 10^{-8} M to 10^{-6} M, are similar to those used in our studies to inhibit growth.

Since the regulation of several enzyme systems which are influenced by insulin have been linked to changes in cyclic AMP levels, it seems possible that insulin might act by altering intracellular levels

of this cyclic nucleotide. Sutherland and co-workers (25) have found that insulin abolishes the increase in liver cyclic AMP in alloxan induced diabetes in rats. They also found that insulin causes a small (22%) decrease in liver cyclic AMP levels in vitro and it counteracts the effects of hormones such as epinephrine and glucagon which act by raising cyclic AMP concentrations. However, other groups found that insulin either had no effect (26) or actually increased cyclic AMP levels in certain cells (27). Jarrett and co-workers (26) found that insulin at several different concentrations caused no change in cyclic AMP levels in rat adipocytes, but greatly stimulated protein synthesis. Insulin also did not alter the elevations in cyclic AMP caused by epinephrine but did overcome the inhibition of protein synthesis caused by this hormone. In this case, insulin appeared to alter protein synthesis rates by a mechanism other than regulation of cyclic AMP levels. Other evidence suggests that the effects of insulin on glucose release from the liver can be dissociated from effects of intracellular cyclic AMP levels (28).

From the results presented here, insulin (10^{-7} M) does not appear to inhibit tyrosinase activity by lowering basal cellular cyclic AMP levels, nor does it inhibit the MSH stimulation of cyclic AMP. It has been shown that MSH has no effect on insulin binding to melanoma cells (15). Our results also suggest that insulin does not interfere with MSH binding, nor does it lower the level of expressed MSH receptors. It is possible, however, that exposure of cells to insulin for periods longer than 30 minutes might be necessary to detect a reduction in cyclic AMP levels.

Insulin has been reported to stimulate Ca^{++} dependent phosphodiesterase activity in fat cell homogenates (29) which may in turn decrease cyclic AMP levels. The studies performed here with the phosphodiesterase inhibitor, theophylline, as well as the cyclic AMP analogue, dibutyryl cyclic AMP, make it seem unlikely that insulin produces its inhibitory effects by altering cellular cyclic AMP levels. We found that insulin was effective in inhibiting tyrosinase activity in cultures exposed to either compound, suggesting that insulin exerts its effects at some point past the production of cyclic AMP. If insulin functioned to inhibit the inducible rise of cyclic AMP, it would be expected that no inhibition of tyrosinase activity would occur with either compound since both cause a large increase in intracellular cyclic AMP levels; also, the proposed site of insulin action would have already been passed with addition of either compound. In contrast to our results, Pawelek and co-workers have shown that insulin lowers endogenous cyclic AMP levels and suppresses the MSH stimulated rise in cyclic AMP (13,30).

Several studies have suggested that insulin may function through cyclic GMP to exert its effects (22,23). Spence and Pitot (31) have shown that addition of insulin (1 μ M) to a culture medium of rat hepatocytes produces a 2-fold increase in glucokinase activity which can be mimicked by 8-Br-cGMP (0.1mM) but not by (Bu)₂ cAMP. Addition of insulin to hepatocytes previously stimulated by 8-Br-cGMP had no effect suggesting that the inductive effects of insulin may be mediated through cyclic GMP. Pinkett and Perlman (32) also found that 8-Br-cGMP mimics insulin action in its ability to increase transport of sugars

into muscle but, unlike insulin, has no effect on the incorporation of glucose into muscle glycogen. Illiano et al. (33) have found that low concentrations of insulin (120uU/ml) increase cyclic GMP in isolated fat cells by 350% within two minutes after exposure. Since it has previously been shown that insulin lowers cyclic AMP levels through inhibition of adenylate cyclase activity (34), it has been suggested that insulin may simultaneously modulate cyclic AMP and cyclic GMP levels to exert its effects. Interaction of insulin with its receptor may transform the substrate specificity of adenylate cyclase into a form that favors GTP. Since the enzyme system synthesizes both cyclic AMP and cyclic GMP, the utilization of ATP or GTP would depend on the occupancy of receptors that favor one form over the other.

In these studies we were unable to mimic insulin action on melanoma cell cultures with either 8-Br-cGMP or (Bu)₂ cGMP at either 10^{-6} or 10^{-9} M. These results indicate that insulin does not exert its inhibitory effect on tyrosinase activity through cyclic GMP, although concentrations of cyclic GMP analogues other than those used here may elicit a different response. It may also be possible that cyclic GMP acts in concert with other factors when exposed to insulin to regulate enzyme activity.

Exposure of mouse melanoma cells in culture to MSH results in a marked increase in tyrosinase activity, a response which is completely suppressed by the addition of either cyclohexamide or Actinomycin D (21). Since these findings indicate an absolute requirement for both RNA and protein synthesis for the MSH stimulation of tyrosinase, it is interesting to speculate that insulin may act by altering either or

both of these metabolic processes. Insulin appears to play an important role in regulating the synthesis of proteins in many tissues; however, it is not known whether it does this by enhancing or inhibiting translation of mRNA that is already present or by changing the amount or activity of that mRNA. We observed a marked reduction in protein synthesis in melanoma cell cultures treated with insulin for 48 hours to a level which corresponded well with the observed reduction of tyrosinase activity. Therefore, this reduction in translational capacity may cause the observed inhibition of tyrosinase activity in cells exposed to insulin. This inhibitory effect on translation has also been observed in another laboratory which describes the insulin mediated reduction of phosphoenolpyruvate carboxykinase (PEPCK) mRNA activity (8). In this study, insulin inhibited the $(\text{Bu})_2$ cAMP-mediated rise in PEPCK mRNA activity at concentrations as low as 10^{-12} M, with maximal inhibition occurring at 10^{-10} M.

The findings presented here which demonstrate the inhibition of tyrosinase by insulin suggest that insulin does not alter cyclic AMP levels nor does it exert its effects through cyclic GMP. The elucidation of insulin action involves the identification of events occurring in response to insulin-receptor interaction. It is not known whether the signal that leads to insulin's many effects involves only the membrane or whether it involves cytoplasmic factors as well. It may also be possible that multiple signals are generated. Although the molecular basis of insulin action remains unknown, several interesting possibilities have currently been described which may represent important new leads. One of these involves the recent discovery of

bioactive peptides released from cell membranes following insulin treatment. It has recently been shown that incubation of purified plasma membranes from rat adipocytes with insulin releases a soluble product which, after centrifugation, can be recovered in the supernatant and can stimulate pyruvate dehydrogenase in isolated mitochondria (35). This effect occurs without added ATP which confirms an earlier suggestion that stimulation of pyruvate dehydrogenase by insulin occurs by the stimulation of phosphatase activity (36). This mediator has been identified as a peptide with a molecular weight of about 2000 daltons (37). There is much evidence that the mediator is formed by proteolysis from plasma membranes. Seals and Czeck (35) have reported that 1) trypsin mimics the action of insulin on plasma membranes 2) protease inhibitors inhibit insulin action and 3) digestion by proteases of the plasma membrane supernatant that contains mediator activity destroys its activity. They have put forth the hypothesis that the binding of insulin to its receptor results in proteolysis of a membrane substrate either by activation of a membrane protease or by increased access of the protease to the substrate. The result is the release of a peptide fragment of the substrate that acts as a mediator of several enzymes such as pyruvate dehydrogenase, possibly by altering phosphatase or protein kinase activity, or both.

Recently, much evidence has been presented suggesting that more than one mediator is generated by the interaction of insulin with the plasma membrane. It has generally been believed that insulin affects enzyme systems by causing a dephosphorylation, but there are now reports of enzymatic systems in which insulin increases phosphorylation.

Larner et al. (38) showed that the Sephadex G25 active fraction from skeletal muscle produced a biphasic response with respect to the activity of glycogen synthase phosphoprotein phosphatase (GSPP). Upon separation of this into two separate fractions, they found that one fraction stimulated cAMP dependent protein kinase and inhibited GSPP. The other fraction inhibited the kinase and increased the phosphatase. Further findings support the idea that two messengers may exist. It has been found that treatment of adipocyte plasma membranes with physiological concentrations of insulin decreases activity and phosphorylation of $(\text{Ca}^{2+} + \text{Mg}^{2+})\text{-ATPase}$ (39). McDonald and co-workers (40) found that the chemical mediator in the supernatant from the adipocyte plasma membrane stimulated this enzyme system and increased calcium transport. When the supernatant was fractionated by Sephadex G25 chromatography only the low molecular weight fraction increased $(\text{Ca}^{2+} + \text{Mg}^{2+})\text{-ATPase}$ and Ca^{2+} transport activity. This effect was opposite to that observed with physiological concentrations of insulin. Since the $(\text{Ca}^{2+} + \text{Mg}^{2+})\text{-ATPase}$ is controlled by phosphorylation (41) the mediator may act by increasing phosphorylation.

Jarret and co-workers propose a model in which two mediators are generated in response to insulin (42). One mediator decreases phosphorylation of enzymes through stimulation of phosphoprotein phosphatase, inhibition of cyclic AMP dependent protein kinase, or both. This includes the glycogen synthase and pyruvate dehydrogenase systems. The second mediator increases phosphorylation through an increase in cAMP dependent and/or independent protein kinase, an increase in Ca^{2+} dependent calmodulin mediated protein kinase, or a decrease in the

activity of phosphoprotein phosphatase. The effect of mediator from adipocytes on $(\text{Ca}^{2+} + \text{Mg}^{2+})$ -ATPase is an example of this response.

Other more recent studies have raised the possibility that the insulin receptor itself is a protein kinase. In a study done by Kasuga and others (43) cultured human lymphocytes and rat hepatoma cells were labelled with ^{32}P orthophosphate. Insulin receptors were purified using a wheat germ agglutinin column and immunoprecipitation by a serum containing antibody against insulin receptor. Immunoprecipitates were analyzed by SDS-PAGE and autoradiography, and it was found that the B-subunit of the insulin receptor was selectively phosphorylated. It was also found that insulin increased this phosphorylation in a dose-dependent manner. It was later found that only the tyrosine residue of the B-subunit was phosphorylated (44), indicating that the insulin receptor is a tyrosine-specific kinase. Similar experiments have been performed in a cell-free system using rat liver plasma membranes (45) which further showed that insulin stimulates the incorporation of ^{32}P into the B-subunit at tyrosine residues only. These results suggest that phosphorylation of the insulin receptor is an early event in insulin action. A simple explanation would be that insulin stimulates the kinase activity of the receptor which then phosphorylates itself and other proteins. These phosphorylated proteins would then mediate various reactions which are responsible for the effects of insulin.

Another interesting possibility for the mechanism of insulin action involves the calcium dependent regulatory protein, calmodulin. it is known that the intracellular concentrations of calcium ions

appears to play a major role in the insulin secretory process. In response to glucose, depolarization of the B cell membrane results in an increase in the intracellular accumulation of calcium ions which appears to trigger the release of insulin (46). How these calcium ions exert their regulatory functions has become clearer with the discovery of the many roles calmodulin plays in mediating calcium dependent cellular processes. Calmodulin was discovered over ten years ago by Wai Yiu Cheung as an activator of brain cyclic nucleotide phosphodiesterase (PDE) (47). It was later found that calmodulin binds Ca^{2+} . It was also found that brain PDE requires Ca^{2+} ions for full activity and that calmodulin increases the enzymes response to the presence of calcium. It is single protein chain consisting of 148 amino acid subunits and is folded into four matching domains, each of which has a calcium binding site. It is a very resilient molecule, being resistant to denaturation by boiling, urea or detergents such as SDS. It is important in several metabolic processes such as glycogen metabolism, cyclic nucleotide metabolism, calcium transport, and protein phosphorylation.

Several studies have been performed which describe the role of calmodulin in insulin action. Pershadsingh and co-workers (48) discovered an ATP dependent Ca^{2+} transport pump in adipocyte plasma membranes which is stimulated approximately three-fold by calmodulin. It was previously found that high affinity $(\text{Ca}^{2+} + \text{Mg}^{2+})$ -ATPase in the same preparation was specifically inhibited by physiological concentrations of insulin (40). This ATP-ase appears to be the functional component of the calcium extrusion pump which is stimulated by

calmodulin. Through these findings it has been theorized that the cell possesses both signals for increasing intracellular calcium levels and for turning these signals off. By inhibiting the ATP-ase, insulin causes an increase in cytoplasmic free Ca^{2+} which may mediate metabolic information via Ca^{2+} or a Ca^{2+} -calmodulin complex. Activation of the Ca^{2+} extrusion pump by calmodulin could provide a means of negative feedback control for these effects. This theory could explain the activation of pyruvate dehydrogenase by insulin mentioned earlier since pyruvate dehydrogenase phosphatase is activated by Ca^{2+} (49).

Ashcroft and others demonstrated the presence of calmodulin in rat pancreatic islets of Langerhans (50), and several studies have been done exploring the effects of calmodulin on insulin secretion. Many of these studies depend upon drugs which bind to calmodulin and inhibit its action, namely trifluoperazine. It binds to two high affinity binding sites on calmodulin and inhibits the Ca^{2+} -calmodulin dependent activation of phosphodiesterase. Gagliardino and co-workers (51) showed that trifluoperazine (5-50mM) and other related drugs produced a dose dependent inhibition of calmodulin dependent activation of phosphodiesterase. Gagliardino and co-workers (51) showed that trifluoperazine (5-50mM) and other related drugs produced a dose dependent inhibition of calmodulin activated phosphodiesterase which paralleled their inhibition of glucose (20mM) stimulated insulin release from rat pancreatic islets, thereby indicating calmodulin's possible role in stimulus-secretion coupling in the B-cell. It is also possible that the effects of calmodulin on insulin secretion could be related to interaction with cyclic AMP or cyclic GMP (52,53).

Calmodulin may be indirectly involved in mediating the effects of insulin via phosphorylation of islet proteins. Ashcroft and others (51) found that incorporation of ^{32}P into islet protein (MW, 55K) was greatly enhanced by addition of Ca^{2+} (1mM) and calmodulin (100ng) using SDS/PAGE to resolve the phosphorylated bands. This enhanced incorporation was abolished by trifluoperazine but not by cyclic AMP dependent protein kinase inhibitor. This evidence suggests that islets contain Ca^{2+} -calmodulin dependent protein kinase activity and substrates for this activity. The phosphorylated proteins would then be involved in mediating the effects of insulin.

These recent studies have contributed much insight into how insulin may be exerting its effects. The mouse melanoma system can also be used to more clearly define the cellular mechanisms of calmodulin, peptide mediators, and even the insulin receptor itself, which may increase our understanding of the mechanism of insulin action.

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