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Neurobiology

Insulin, insulin-like growth factor II, and nerve growth factor effects on tubulin mRNA levels and neurite formation

(actin mRNA/axons/differentiation/human neuroblastoma)

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ABSTRACT **We have previously shown that insulin and the insulin-like growth factors share some important neurotrophic properties with nerve growth factor (NGF), including the capacity to enhance neurite formation. In this study, we have examined the effects of these neuritogenic agents on the expression of genes coding for important cytoskeletal proteins of axons and dendrites. Insulin specifically and coordinately increased the levels of α- and β-tubulin mRNAs in human neuroblastoma SH-SY5Y cells. The dose–response curves for these increases were very similar to that for enhancement of neurite formation. Tubulin transcripts reached a transient maximum in ≈1 day, suggesting that higher levels are important during initiation of neurites and that high levels are not required to sustain neurites once formed. Insulin-like growth factor II shared with insulin the capacity to substantially increase tubulin mRNA levels. NGF had but a small effect. Complementary mechanisms for these neurotrophic agents are suggested, because other studies show NGF and insulin can synergistically potentiate neurite formation. None of the factors altered the levels of actin mRNA. Thus, neurite formation does not seem to require a coordinate increase in actin and tubulin transcripts in SH-SY5Y cells.**

Emerging evidence suggests that insulin and insulin-like growth factor II (IGF-II) have neurotrophic properties. Neurite formation is stimulated by physiological concentrations of these factors in sensory, sympathetic, and human neuroblastoma cells (1–3). The survival of embryonic neurons is also supported (2, 3). The insulin-like factors, thus, have significant properties in common with nerve growth factor (NGF), the classic neurotrophic agent. Reviews on the role of NGF in the development of the vertebrate nervous system are available (4–6). The IGF-II gene is expressed in fetal rat brain (7), and binding sites for insulin (8, 9) and IGF-II (10) are present in brain homogenates, supporting the conviction that these factors play an important part in the central, as well as the peripheral, nervous system.

We are concerned with the cellular and molecular mechanisms whereby physiological effectors do regulate neurite formation. Insulin, IGF-II (1), and NGF (11, 12) can stimulate neurite formation in the cloned (13) human neuroblastoma SH-SY5Y cell. The neurites have an ultrastructure similar to that of developing sympathetic ganglion cells, and end in structures typical of neuronal growth cones (14). Veratridine-dependent Na^+ uptake is additionally increased by NGF (11). These cells, then, provide a model system in which to explore neurite outgrowth under regulation by multiple neurotrophic factors. The neuritogenic agents mentioned above may activate common biochemical events implicated in neurite formation (2). The regulation of tubulin mRNA levels by physiological effectors has, in general, not been studied. Here, we investigated, in SH-SY5Y cells, whether insulin, IGF-II, and NGF could share common effects on the levels of the mRNAs coding for actins and tubulins, which are cytoskeletal proteins important for the structure and function of axons. The α- and β-tubulins have distinct amino acid sequences and are coded for by different mRNAs (15). The roles of actin and tubulin in axonal structure (16), and microfilaments and microtubules in axonal transport (17), have been described.

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MATERIALS AND METHODS

Materials. Porcine pancreatic insulin (24 units/mg) was purchased from Sigma, dissolved in 0.01 M HCl, and stored at −20°C. Rat IGF-II, also known as rat liver cell multiplication-stimulating activity (18), was prepared as described by Moses *et al.* (19) and was kindly provided by Matthew M. Rechler. The β subunit of NGF was prepared from male mouse saliva (20); purity was confirmed by the presence of a single band on isoelectric focusing (pH, 3.5–10.0) in 7.5% polyacrylamide gels, and $NaDodSO_4$ gel electrophoresis. Activity was determined by bioassay (21). The cDNA clone pKα1 contains the entire α-tubulin coding region plus 67 and 173 base pairs of the 5′ and 3′ nontranslated regions, respectively (22). The cDNA clone pDβ1 contains all but 21 bases from the 5′ end of the coding region for β-tubulin, and 141 bases in the 3′ untranslated region (23). The cDNA clone pHF1 contains the entire 3′ noncoding region and the coding region of γ-actin beyond that for amino acid 144 (24). The human cDNA clone pKα1 was the kind gift of Donald W. Cleveland, and pDβ1 was generously provided by Nickolas Cowan. The human neuroblastoma cell line SH-SY5Y (13) was a kind gift from June L. Biedler. Cells between passage numbers 9 and 29 were studied.

Neuroblastoma Cell Cultures. Cells were maintained in the logarithmic phase of growth in plastic tissue culture flasks in RPMI 1640 medium supplemented with 12% fetal calf serum, 50 units of sodium penicillin-G per ml, and 25 μg of streptomycin sulfate per ml at 37°C in humidified 5% CO_2/95% air. SH-SY5Y cells were passaged after treatment with 0.05% trypsin and 1 mM EDTA in Hanks' salts solution and dispersion by trituration (12, 25).

Neurite Outgrowth. The method has been described (1). Briefly, a single cell suspension was prepared by treatment with trypsin to discourage the formation of cell aggregates, which sometimes can obscure neurites. To permit recovery from the trypsin treatment, test solutions were added only after several days under normal culture conditions. Insulin

Abbreviations: IGF-II, insulin-like growth factor II (rat liver multiplication-stimulating activity); NGF, β nerve growth factor from mouse submaxillary gland; kb, kilobase(s).

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enhanced neurite formation to the same extent whether cells were plated after trypsin or EDTA treatment. For studies in serum-free media, after attachment, cells were washed three times with warm RPMI 1640 medium, and then fresh warm RPMI 1640 medium containing various test solutions was added. The percentage of cells bearing neurites was determined by replicate counts on >100 cells in several randomly chosen fields under low-power modulation contrast microscopy. Only cells with processes equal to or longer than 40 μm were scored as positive. Cells with multiple processes were scored only once. Cell aggregation sometimes obscured neurites and aggregates were not scored. Neurites could generally be identified by the presence of growth cones. Values are means ± SEM; *n* values indicate the number of replicate cultures.

RNA Preparation. Cells were seeded and permitted to attach for several days. In the case of those experiments conducted in serum-free medium, cultures were washed twice with RPMI 1640 medium and incubated for 2 days in RPMI 1640 medium prior to addition of ligands. Other conditions are described in the individual figure legends. For each experimental point, 4–6 dishes, each containing 1–2 × 10^7 cells, were used. The dishes were always <50% confluent. Cells were harvested by trituration in cold serum-free medium and washed once in 15 ml of cold serum-free medium. RNA was immediately extracted from the cell pellet and purified by the guanidine isothiocyanate/cesium chloride method (26). The same method was used to prepare RNA from the brains of fetal and adult Wistar rats. RNA concentrations and purity were estimated on the basis of optical density measurements at 260 and 280 nm.

RNA Electrophoresis and Hybridization to cDNA. Equivalent amounts of RNA (25 or 30 μg), determined by optical density, were electrophoresed through 0.8% agarose gels containing formaldehyde, as described (27). Ethidium bromide staining was used to determine the position of 18S and 28S ribosomal RNAs and, additionally, confirmed that equivalent amounts of RNA had been loaded. The RNA was transferred to nitrocellulose (28), and the blots were hybridized to the nick-translated (29) ^{32}P-labeled cDNA probes for 16–18 hr at 42°C. The blots were washed for 30–40 min each with 2× NaCl/Cit at 42°C, 2× NaCl/Cit at 68°C, twice with 1× NaCl/Cit at 68°C, and 0.5× NaCl/Cit at 68°C (1× NaCl/Cit is 150 mM NaCl/15 mM sodium citrate, pH 7). Autoradiograms were prepared on x-ray film by using enhancing screens at −70°C. The autoradiograms were scanned on an EC910 densitometer (E. C. Apparatus, St. Petersburg, FL) that was connected to a strip chart recorder. The area under the curve was cut out and weighed. Boiling for 5 min in water was sufficient to remove all hybridized cDNA, as indicated by autoradiography, and nitrocellulose blots were rehybridized with each of the cDNA probes in sequence to obtain the parts shown in the individual experiments.

RNA Dot Blot Hybridizations. Dot hybridization of cytoplasmic RNA was performed as described (30). Equivalent numbers of cells were used in comparisons. The optimum cell concentration was determined by dot hybridization with cytoplasm from 1–5 × 10^5 cells on nitrocellulose filters. The autoradiograms were scanned on a densitometer.

Dot hybridization of total RNA was carried out as follows. RNA was boiled in H_2O for 5 min, then diluted with an equal volume of 20× NaCl/Cit and placed on ice. Equivalent amounts of RNA (3 μg) were blotted in 100-μl aliquots onto nitrocellulose filters on the Bio-Dot microfiltration apparatus (Bio-Rad). The autoradiograms were scanned on a densitometer. Alternatively, the dots were punched out and the radioactivity was counted in a liquid scintillation spectrometer.

RESULTS

Insulin, IGFs, NGF, and other factors in the serum component of the culture medium may alter the level of spontaneous neurites in SH-SY5Y cultures (31). To avoid this complication, some tests were conducted in serum-free medium, in which SH-SY5Y cells can survive at least a week without loss in number (12). Growth resumes on the reintroduction of serum. Insulin and IGF-II can reversibly enhance neurite formation in serum-free medium (1).

Tubulin mRNA Levels in SH-SY5Y Cells and Rat Brain. RNA was extracted from SH-SY5Y cells, some cultures of which were treated with insulin for 1 day in serum-free medium. For purposes of comparison, RNA was also extracted from the brains of adult and 18-day-old embryonic rats. Equivalent amounts of RNA were electrophoresed on 0.8% agarose gels containing formaldehyde. After transfer to nitrocellulose filters, the RNA was hybridized to the ^{32}P-labeled pKα1, pDβ1, and pHF1 human cDNA probes. These probes contain all, or much, of the gene-coding regions. Thereafter, autoradiograms were prepared. As shown in Fig. 1 (lanes 1 and 2), insulin increased the content of α- and β-tubulin mRNAs in SH-SY5Y cells. Only a single α-tubulin mRNA size class of 1.8 kilobases (kb) was observed. Two β-tubulin mRNA bands of 1.8 and 2.6 kb were seen, both of which were increased by insulin.

For purposes of comparison, rat brain RNA was examined. Heterologous cDNA probes cross-hybridize readily because of the substantial evolutionary conservation of tubulin gene-coding regions. The content of tubulin mRNA was greatly diminished in the adult relative to the 18-day-old fetal rat brain (Fig. 1, lanes 3 and 4). This developmental pattern agrees with the earlier observation of Bond and Farmer (32). The level of expression of α- and β-tubulin mRNAs in SH-SY5Y cells is high, resembling more that in embryonic than in adult rat brain. The levels remain high after differentiation caused by insulin. The pattern differs, however, in that the 2.6-kb β-tubulin mRNA is expressed at a level nearly equal to that of the 1.8-kb species in SH-SY5Y cells, whereas

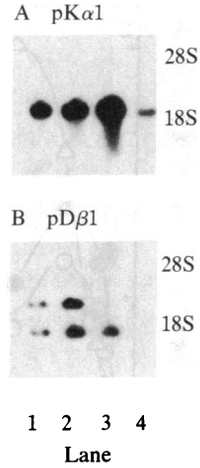

FIG. 1. Effect of insulin on α- and β-tubulin mRNA levels in SH-SY5Y cells, and comparison with levels in fetal and adult rat brain. Human neuroblastoma SH-SY5Y cells were incubated with 0.1 μM insulin for 1 day in serum-free medium. The cultures were harvested, total RNA was extracted, and 25 μg of RNA per lane was electrophoresed on 0.8% agarose gels that contained formaldehyde. Rat brain RNA was extracted and processed in the same manner. The RNA was transferred to nitrocellulose filters. Autoradiogram of a nitrocellulose filter hybridized to the ^{32}P-labeled α-tubulin cDNA probe, pKα1 (*A*) and to the ^{32}P-labeled β-tubulin cDNA probe, pDβ1 (*B*). Lanes: 1, untreated SH-SY5Y cells; 2, insulin-treated SH-SY5Y cells; 3, embryonic 18-day-old rat brain; 4, adult rat brain.

in embryonic rat brain the latter clearly is predominant. The sizes of mRNA species were established from the positions of 18S and 28S rRNAs visualized by ethidium bromide staining and the position of ^{32}P-labeled *Hin*dIII digests of λ phage DNA.

Total RNA from several experiments was hybridized on a single filter and analyzed by using a dot-blot procedure (28, 33) to quantify the increases. After hybridization to cDNA probes, the dots were punched out and the radioactivity was measured. After 1 day of 0.1 μM insulin treatment, there was a significant difference ($P = 0.05$) in the amount of bound cpm per assay between untreated and insulin-treated groups for both α- and β-tubulin mRNAs. The values for pKα1 were as follows: untreated, 93 ± 11; treated, 195 ± 9. The values for pDβ1 were as follows: untreated, 98 ± 10; treated, 141 ± 14. The values are means ± SEM, where n = five experiments with four replicate determinations of each value. No significant difference was observed with respect to actin mRNA levels. When analyzed on a per 10^6 cell basis, the increases were greater because insulin increases the content of SH-SY5Y cell RNA (data not shown). It is clear that the increases observed on the RNA blots and total RNA dot blots are specific because these results are expressed relative to the content of total cellular RNA. Moreover, the level of γ-actin mRNA was not increased (see also Figs. 2, 4, and 5).

Time Course of the Effect of Insulin on Tubulin and Actin mRNA Levels in Serum-Free Medium. To study whether insulin provokes a coordinated increase, and to determine the relationship to neurite formation, the time course for the accumulation of α- and β-tubulin mRNAs was examined in serum-free medium (Fig. 2). In some experiments, the levels of α- and β-tubulin mRNAs were found to peak together at ≈24 hr (Fig. 2 *A* and *B*). However, in a separate experiment, the 1.8- and 2.6-kb β-tubulin mRNAs peaked somewhat earlier than the α-tubulin mRNA levels (Fig. 2*C*). We believe this observation is not an artefact, because the results were obtained from the same nitrocellulose blot by boiling and rehybridizing to a different ^{32}P-labeled cDNA probe. In this and other experiments, the -fold increase in α-tubulin mRNA levels at the peak was substantially greater than that for either the 1.8- or 2.6-kb β-tubulin mRNA levels. It is interesting, however, that, in all time course experiments, the sum of the two β transcripts at the peak attains a level quite close to that of the α-tubulin mRNA. The overall pattern, then, indicates coordinate expression of α- and β-tubulin transcripts. In all studies, there was a clear decrease in the levels of each of the tubulin mRNAs subsequent to the peak, the consequences of which shall be discussed.

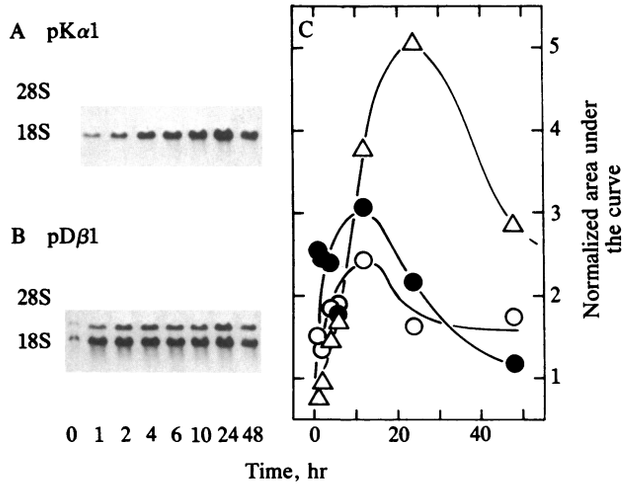

FIG. 2. Time course of the effect of insulin on levels of tubulin mRNAs in SH-SY5Y cells. The cells were incubated in serum-free medium with 0.1 μM insulin for various lengths of time as shown. RNA was extracted and processed as described in Fig. 1. (*A*) Autoradiogram of a filter hybridized to ^{32}P-labeled pKα1. (*B*) The same filter rehybridized to radioactive pDβ1. (*C*) In a separate experiment, the autoradiograms, prepared as in *A* and *B*, were scanned on a densitometer, and the area under each curve was measured. The plots show the normalized area under the curve for each experimental point relative to untreated cultures. △, α-Tubulin mRNA; ○, 1.8-kb β-tubulin mRNA; ●, 2.6-kb β-tubulin mRNA.

The kinetic pattern of tubulin transcript expression was confirmed by using the cytoplasmic dot hybridization method. The results demonstrated clearly that cytoplasmic tubulin mRNA levels are increased by insulin (data not shown). The cytoplasmic hybridization procedure yielded results that showed larger increases than the RNA blot method, because the results of the former are expressed on a per cell basis and do not take into account the increase in overall RNA content caused by insulin treatment.

The same RNA blot filters described above were rehybridized to cDNA probe pHF1. The effects on actin mRNA levels were somewhat variable. In one experiment, the transcript levels were transiently increased ≈1.5-fold at 4 and 6 hr. However, a transient peak was not observed in two other experiments. Overall, we have no clear evidence that actin mRNA levels are increased.

Relationship Between Insulin Concentration, Tubulin mRNA Levels, and Neurite Outgrowth. SH-SY5Y cells were incubated with various concentrations of insulin for 1 day in serum-free medium. The densitometric scans of the autoradiograms, normalized to the maximum response for α- and β-tubulin mRNA levels, are shown in Fig 3*A*. The half-maximum responses were at ≈1 and ≈3 nM for α- and β-tubulin mRNAs, respectively. The close correspondence in the curves for α- and β-tubulin mRNAs reinforce further the suggestion of coordinate expression of transcripts.

The relationship between insulin concentration and neurite outgrowth is shown in Fig. 3*B*. The shape of the curve is the same as shown earlier, where neurite outgrowth is increased half-maximally at 4 nM (1). Thus, the dose–response curves for enhanced neurite outgrowth and for increased levels of tubulin mRNAs are very similar. Physiological concentra-

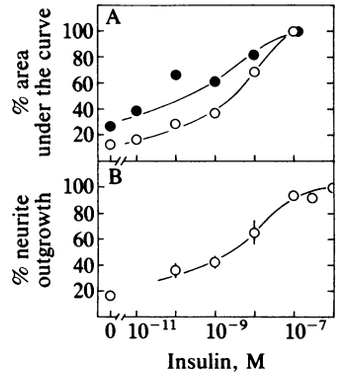

FIG. 3. Dependence of tubulin mRNA levels on insulin concentration and relationship to neurite formation in SH-SY5Y cells. (*A*) Cells were incubated in serum-free medium for 1 day with various concentrations of insulin as shown and then analyzed for tubulin mRNA content as described in Fig. 2. The area under each curve is shown as a percentage of the maximum response for α-tubulin (○) and β-tubulin (●) mRNAs. For β-tubulin, the values are the sum of the 1.8- and 2.6-kb mRNAs. (*B*) In a separate experiment, the cells were incubated in serum-free medium with various concentrations of insulin and the proportion of cells with neurites was scored. Values are means ± SEM of three cultures. Percentages are relative to the maximum response.

tions of insulin are active. The probable cause of the broad dose–response curves for these functions is discussed.

Effect of NGF, IGF-II, and Serum on Tubulin and Actin mRNA Levels. NGF can enhance neurite formation in SH-SY5Y cells cultured in serum-containing (11, 12), but not in serum-free (31) medium. SH-SY5Y cells lose the capacity to bind and respond to NGF under the latter conditions. By itself, NGF enhanced slightly the level of α- and β-tubulin mRNA in serum-free medium (Fig. 4, lane 1). The addition of 0.1 nM insulin to serum-free medium maintains the capacity of SH-SY5Y cells to bind and respond to NGF (31). This low concentration of insulin by itself has but a small effect on neurite formation (31) and tubulin mRNA levels (lane 2). The combined effects of NGF and the low concentration of insulin was greater than either treatment alone (lane 3), but it was far short of the response caused by an optimum concentration of insulin (lane 5).

It was possible that the response to NGF requires other serum factors, and a test was conducted in serum-containing medium (Fig. 5). Insulin increased tubulin mRNA levels (lane 2), but the relative increase was not as robust as in serum-free medium. This is because the basal levels of mRNAs and spontaneous neurite outgrowth are higher, most likely because of the presence of neuritogenic agents and other factors in serum. NGF by itself appeared to provoke a small increase (lane 3). The combination of insulin and NGF was no better than either factor alone (lane 4). In these studies, the concentration of NGF used was sufficient to produce a maximum neurite outgrowth response (12).

IGF-II and insulin are structurally related polypeptides with overlapping actions (34, 35). The effects of IGF-II were studied in serum-free medium. IGF-II greatly increased the levels of α- and β-tubulin mRNAs (Fig. 5, lanes 5 and 6). The dose of IGF-II studied is within the range of circulating concentrations (36). Neither insulin, IGF-II, nor NGF was observed to increase actin mRNA levels, whether studies were conducted in serum-free (Fig. 4) or serum-containing (Fig. 5) medium. Thus, these neuritogenic agents all increased tubulin, but not actin, mRNA levels.

DISCUSSION

Our results show that insulin, IGF-II, and, to a lesser extent, NGF share the capacity to specifically enhance tubulin mRNA levels. Physiological concentrations of insulin can coordinately increase the levels of α- and β-tubulin mRNAs.

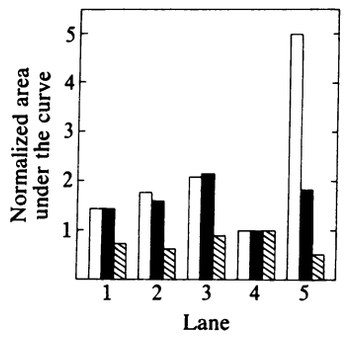

FIG. 4. Effect of NGF, insulin, and their combination on the levels of tubulin and actin mRNAs in cells cultured in serum-free medium. The cells were incubated for 1 day in serum-free medium with various neuritogenic agonists, then the content of α-tubulin, β-tubulin, and actin mRNAs was measured. The autoradiograms were scanned with a densitometer. The figure shows the area under each curve normalized to that for untreated cultures. Lanes: 1, 4 nM NGF; 2, 0.1 nM insulin; 3, 4 nM NGF and 0.1 nM insulin; 4, untreated; 5, 0.1 μM insulin. Open bars, α-tubulin mRNA; solid bars, β-tubulin mRNAs (both bands); striped bars, actin mRNA.

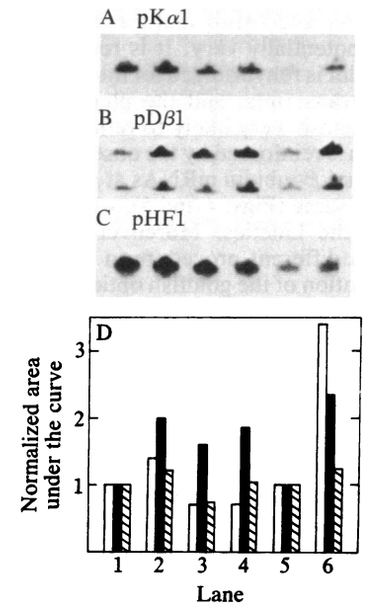

FIG. 5. Effect of insulin and NGF on content of tubulin and actin transcripts in cells cultured in serum-containing medium, and effect of IGF-II in serum-free medium. Cells were cultured for 1 day in medium containing 12% fetal calf serum and the following additions: lane 1, no additions; lane 2, 0.1 μM insulin; lane 3, 4 nM NGF; lane 4, 0.1 μM insulin and 4 nM NGF. Cells were cultured for 1 day in serum-free medium as follows: lane 5, no addition; lane 6, 10 nM IGF-II. (*A–C*) Autoradiograms; (*D*) normalized area under the curve values relative to untreated cultures. Open bars, α-tubulin mRNA; solid bars, β-tubulin mRNA (both bands); striped bars, actin mRNA.

The capacity to enhance the content of tubulin transcripts is correlated closely with the ability of these agents to enhance neurite formation in SH-SY5Y cells. The 2- to 4-fold increases observed are truly substantial when one considers that tubulin mRNA already comprises a major fraction of the total transcript pool in the unstimulated cell and that most of the increased activity is probably directed predominantly toward neurite formation.

Within 1 or 2 hr of addition of insulin, tubulin mRNA content is already increased (Fig. 2). The tubulin mRNA levels appear to attenuate after ≈1 day of exposure, or less, to insulin. The neurite outgrowth is near the maximum after 1 day in serum-free medium. However, in contrast to the tubulin transcript levels, the level of neurites is thereafter sustained (1). The attenuation suggests that the expression of transcripts may be lower after differentiation. A high level of tubulin mRNAs may be important during the initiation of neurite extension. After outgrowth, lower levels may be sufficient to sustain the neurite. Support for this view is obtained in the postnatal rat, where a sharp decline in the production of α- and 1.8-kb β-tubulin mRNAs is observed (32), whose time course is coincident with the period of terminal differentiation and neurite outgrowth in brain. Alternatively, the impact of the changing ratios of brain cell populations, changes in the rate of cell multiplication, and other factors, may not be dismissed. What is the mechanism responsible for the subsequent down-regulation of tubulin transcripts in SH-SY5Y cells? The important studies of Cleveland *et al.* (37) suggest that the cellular concentration of the free tubulin pool may feed back to modulate the level of tubulin mRNAs.

All of our results show a coordinate expression of α- and β-tubulin transcripts in the sense that both are increased by neuritogenic agents, and both are down-regulated. Kinetically, however, in one experiment (Fig. 2*C*) the β-tubulin mRNA content was observed to peak prior to that for

α-tubulin mRNAs, suggesting that the rate of message accumulation may potentially vary. It is recalled that the same nitrocellulose blot is rehybridized for measurement of both α- and β-tubulin transcripts, and the phase shift in the time course experiment is very likely to reflect a real difference. This impression is reinforced by the observation that the -fold increase in α- and β-tubulin mRNAs at a given time of assay is often not the same (Figs. 4 and 5), which may result from differences in the kinetics. Moreover, these findings are confirmed by a different procedure in the dot blot studies. During regeneration of the goldfish optic nerve, the β-tubulin levels are reported to increase more than the α-tubulin levels (38). In addition, in the developing rat brain, the α- and β-tubulin mRNA levels decline with separate rates (32).

Insulin can increase the proliferation rate of SH-SY5Y cells cultured with serum (1). However, insulin increases tubulin mRNA levels and neurite outgrowth whether serum is present or not. Because the cells are essentially quiescent in serum-free medium, there is no correlation of these effects with the cell population growth rate. This is consistent with the lack of correlation between growth rates and differentiation caused by NGF (12) and tumor promoters (25) in these cells.

In SH-SY5Y cells, insulin and IGF-II act through pathways distinct from that of NGF. Only the high affinity slow-type NGF sites are present (12, 39), and neither insulin nor IGF-II can occupy these sites (1). The cells have separate insulin and IGF receptors (unpublished observations). At low ligand concentrations (nM), insulin and IGF-II stimulate neurite outgrowth predominantly through occupancy of their own receptors. IGF receptors have been categorized as types I and II (40). Neurite outgrowth correlates best with occupancy of the type I sites by IGFs in SH-SY5Y cells. We show that low concentrations (nM) of insulin and IGF-II can increase tubulin mRNAs (Fig. 3). Increased tubulin concentrations may promote microtubule formation associated with neurite ourgrowth. At high ligand concentrations, cross-occupancy of receptors can occur. This probably explains the broad dose–response curves for neurite outgrowth (Fig. 3*B*; ref. 1) and tubulin mRNA accumulation (Fig. 3*A*).

We suggest that insulin and IGF-II can cooperate with NGF in neurite formation. The effect of NGF on tubulin mRNA levels is minimal in SH-SY5Y cells, although it is a strong agonist for neurite formation. NGF-induced neurite outgrowth in sensory and sympathetic cells is attended with excess production of microtubules (4). After exposure to NGF for 3 weeks, the microtubules become more resistant to depolymerization with colchicine in PC12 cells (41). It, therefore, seems plausible that a major action of NGF is to increase the assembly and/or stabilization of microtubules from a preexisting tubulin pool. It is evident that insulin and NGF can cooperate in neurite formation, because the combination of these factors can synergistically potentiate neurite formation in SH-SY5Y and rat pheochromocytoma PC12 cells (31). The cooperation does not appear to arise from significant additive or synergistic effects of the combination of factors on tubulin mRNA levels in SH-SY5Y cells (Figs. 4 and 5). Rather, the synergistic potentiation between insulin and NGF may involve a composite effect arising from the greater tubulin mRNA levels due largely to insulin and/or IGFs and from enhanced microtubule formation mediated by NGF. The cooperation, furthermore, may include activation of NGF receptors by insulin and IGFs (31). The suggestion of cooperation is supported by the observation that insulin and NGF can act on the same, or overlapping populations, of sensory and sympathetic neurons (2, 3).

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