Nerve Growth Factor-Induced Increase in [^{3}H]Thymidine Incorporation into Pancreas of Young Rats and Its Partial Blockade by Propranolol or Partial Sialoadenectomy (43079)

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Abstract. **Administration of nerve growth factor (NGF) twice daily for 2 days to rats 11 days at the time of the initial injection resulted in a 6.6-fold increase in [^{3}H]thymidine levels of pancreas, when comparison was made to levels of untreated controls. Isoproterenol (ISO), a β-adrenergic agonist known to produce marked increase in [^{3}H]thymidine incorporation into DNA of salivary glands, caused increases in levels of [^{3}H]thymidine in pancreas that were similar in magnitude to those induced by NGF. The combined administration of ISO and NGF did not cause any increase above those observed with either agent alone. Administration of propranolol (3 mg/kg body wt) prior to administration of ISO prevented the usual ISO-induced increase in DNA synthesis, but propranolol in either a 3- or 9-mg/kg body wt dose, caused only a 50% inhibition of NGF-induced thymidine incorporation. In the absence of the submandibular-sublingual glands, the ISO failed to induced the usual high levels of thymidine incorporation, whereas NGF induced the same high levels observed in rats with submandibular glands intact. NGF did not alter the distribution of β_1- and β_2-adrenoceptors in the pancreas but did increase norepinephrine when the initial administration was at age 5 days, but not when it was given at age 10 days. Since NGF increased DNA synthesis in the absence of submandibular-sublingual glands, whereas ISO did not, this suggests that ISO requires NGF to induce β_1-activation and subsequent synthesis and that NGF is a direct activator.**

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Isoproterenol (ISO) markedly enhances DNA synthesis in the pancreas of young rats but not in the pancreas of adult rats (1). The cause of this difference is ascribed to the different proportion of β_1- and β_2-adrenoceptors present in the pancreas of the two age groups. The pancreas of young animals has at least 27% β_1-adrenoceptors whereas that of adult rats has about 5% (2). Since the growth factors, nerve growth factor and epidermal growth factor (NGF and EGF, respectively) (produced by rat submandibular gland) (3–6), also cause an increased DNA synthesis of parotid and submandibular glands that is dependent at least in part on activation of β_1-adrenoceptors (7), it was of interest to determine whether these factors also affect pancreatic DNA synthesis in young rats and if so to what extent the NGF-induced growth resembles that induced by ISO both in magnitude and in β_1-adrenoceptor mediation. In addition, ISO apparently has little or no stimulatory effect on DNA synthesis in the parotid gland in the absence of the submandibular glands (8), but NGF does (3, 4); whether or not NGF-induced DNA synthesis in pancreas is independent of the presence of the submandibular was determined. Existence of similar growth patterns in the exocrine pancreas in response to NGF would show that these factors have a more general effect that includes growth of exocrine organs other than salivary glands. It is possible that the growth factors themselves may affect the distribution of β-adrenoceptors (since ISO has such an effect in adult (9). Therefore, the number of β-adrenoceptors and distribution of their subtypes were examined in the pancreas following administration of growth factors.

Finally, NGF affects growth of the sympathetic innervation (10, 11). Norepinephrine concentration

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[NE] in the pancreas following NGF treatment was used as a criterion of NGF effects on nerve growth. EGF effects on [NE] as well as distribution of autonomic receptors was also examined to see if a discrimination in effects of the growth factors on the innervation is apparent in this organ.

Materials and Methods

Long-Evans rats, 5–18 days of age, were used in these experiments. They were maintained on Purina chow and water *ad libitum* or permitted to continue suckling, with liquid food (whole milk) available in addition to the milk provided by suckling. The submandibular-sublingual glands were removed under ether anesthesia from some rats; other rats were sham operated while others remained intact. Several litters of the same age were used for a given experiment, and they were also size and sex matched. At least four rats from a given litter of eight (reduced to this litter size at birth) were used for one experimental procedure and four others for another. All animals from a single litter were used at once. All of these groups were injected intraperitoneally twice daily for 2 days with *dl*-isoproterenol in a dose of 50 mg/kg body wt. Nerve growth factor was given intraperitoneally twice daily in a dosage of 10 ng/kg body wt. Some groups were injected with propranolol 20 min before administration of either ISO or NGF; dosage of this β-antagonist was either 3 or 9 mg/kg body wt. Controls were injected with 0.9% saline in volumes corresponding to that used with ISO or propranolol (namely, 0.1 ml). In the morning of the third day, all groups were injected with [^{3}H]thymidine (50 μCi/100 g rat). A portion of pancreas was removed 5 hr later, weighed rapidly on a torsion balance, and placed in Tris-HCl buffer for monitoring of [^{3}H]thymidine incorporation. Samples (100 μl) of tissue (homogenized at 4°C) were removed for precipitation with trichloroacetic acid on glass fiber filters, followed by the addition of scintillation cocktail for measuring [^{3}H] thymidine incorporation. Part of the sample was removed for a protein assay. Duplicates of untreated animals were used to determine basal rates of synthesis. Gland homogenates were prepared by centrifugation at 250*g* for 10 min (4°C) to remove connective tissue.

Homogenates of pancreas for both [^{3}H]quinuclidinylbenzilate (QNB) and [^{3}H]dihydroalprenolol (DHA) binding were prepared by centrifugation at 20,000*g* for 30 min (4°C). The pellet containing the membrane fraction was resuspended in 100 μM dithiothreitol. Membranes were resuspended by a combination of vortex vibration followed by Dounce homogenization. Protein concentrations were subsequently determined by a modification of the Lowry protein assay using bovine serum albumin as standard (12). Binding of [^{3}H]QNB and [^{3}H]DHA was linearly dependent on membrane concentration within this dilution. Binding assays were performed in duplicate using 1.0 ml of diluted membrane and 1.0 nm of [^{3}H]QNB or [^{3}H]DHA. The reaction mixture was incubated for 90 min at 37°C and terminated by the addition of 3 ml of ice-cold 0.9% NaCl. Quantitation of binding was performed by precipitation of membranes from the above slurry onto glass fiber filters, washed three times with five volumes of cold phosphate-buffered saline, and counted for radioactivity by liquid scintillation. Nonspecific binding for QNB was determined by the inclusion of 1.0 μM atropine 10 min prior to addition of labeled QNB. Nonspecific binding for DHA was determined by the inclusion of 10 μM(−)propranolol prior to addition of radiolabel.

For determination of NE, tissues were first homogenized (using a Brinkman Polytron) in 2 ml of 0.05 *M* cold perchloric acid which contained sodium metabisulfite (10 mg/liter) as an antioxidant. Following centrifugation at 3°C, the catecholamines in the protein-free supernatant were adsorbed with alumina at pH 8.6 in a 3 *M* Tris buffer. After washing with water, the catecholamines were eluted with 0.1 *M* perchloric acid.

NE was analyzed using a modification of a method described by Krstulovic (13) employing high-performance liquid chromatography and electrochemical detection. The separation was achieved with a Supelco $C_{18}RP_3$ μm column and a Waters pump. The mobile phase, a phosphate-citric acid buffer, contained sodium acetyl sulfate as an ion pair and methanol as a modifier. The eluting compounds were detected with a Bioanalytical Systems ampermometric detector set at a potential of +0.5 V. Student's *t* test was used for statistical analysis of the data.

Results

The data in Figure 1 show [^{3}H]thymidine incorporation into the pancreas of young rats (11–13 days of age during the experimental manipulations). Administration of NGF twice daily for 2 days led to a 6.6-fold increase in [^{3}H]thymidine levels of pancreas when comparison was made to those of untreated rats. This closely resembled the 7-fold increase observed following 2 days of ISO administration. Simultaneous administration of NGF and ISO caused no increase from values observed with either agent alone (Fig. 1). When removal of the submandibular-sublingual (SM-SL) glands preceded initiation of administration of NGF, the [^{3}H]thymidine levels were nearly as high as those observed in intact rats given NGF (6.2-fold increase over controls). On the other hand, in the absence of the SM-SL, the ability of ISO to induce DNA synthesis was markedly depressed, and [^{3}H]thymidine levels of the desalivated ISO-treated pancreas were 29% of those of ISO-treated rats with intact SM-SL glands. Desalivation itself, as expected, did not affect [^{3}H]thymidine incorporation and values did not differ from those of control rats (Fig.

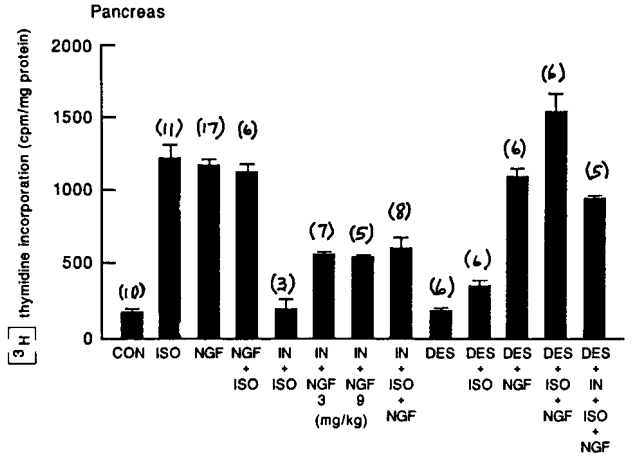

Figure 1. Values are mean ± SE for the pancreas of 13-day-old rats. Con, no drugs administered, glands intact; ISO, i.p., *dl*-isoproterenol HCl, 50 mg/kg body wt, two times daily; NGF, i.p., NGF, 10 ng/kg body wt, two times daily; ISO + NGF, both drugs given i.p. in doses above; IN + ISO, IN = propranolol, given 20 min prior to ISO, in a dose of 3 mg/kg body wt; IN + NGF, propranolol given 20 min prior to NGF in a dose of 3 or 9 mg/kg body wt; IN + ISO + NGF, dosages as described above; DES, partial desalivation involving removal of both submandibular-sublingual glands; DES + ISO or NGF, drugs administered in doses described above, beginning 2 days after desalivation. Numbers in parentheses equal number of rats. Values for ISO, NGF, and NGF + ISO differ significantly from controls ($P < 0.05$) but not from each other; value for IN + ISO does not differ significantly from Con ($P > 0.05$) but does differ significantly from values for ISO, NGF, and NGF + ISO; values for IN + NGF do not differ significantly from each other for the 2 doses of IN (3 and 9 mg/kg) ($P > 0.05$), nor are these values different from IN + ISO + NGF, but all values including IN + NGF are statistically different from IN + ISO. Values for DES do not differ significantly from Con ($P > 0.05$), but do differ from DES + ISO, DES + NGF, DES + ISO + NGF, and DES + IN + ISO + NGF ($P < 0.05$); DES + ISO differs from DES + NGF ($P < 0.05$), from DES + ISO + NGF, and from DES + IN + ISO + NGF ($P < 0.05$); DES + NGF and DES + IN + ISO + NGF do not differ from each other ($P > 0.05$) but NGF + ISO differs significantly from DES + ISO + NGF ($P < 0.05$). NGF does not differ significantly from DES + NGF ($P < 0.05$) but ISO differs significantly from DES + ISO ($P > 0.05$).

1). However, when ISO and NGF were administered to the same partially desalivated rats, levels were 24% greater than those observed in intact rats given ISO and 34% greater than those of pancreas of desalivated rats given both ISO and NGF. Thymidine values of pancreas of desalivated rats given both ISO and NGF were 36% higher than those of intact rats given ISO + NGF.

When propranolol was given 20 min prior to administration of ISO, the thymidine incorporation observed in pancreas after 2 days of drug administration was virtually identical to that of controls. However, propranolol, in either a 3- or 9-mg/kg body wt dose, given 20 min prior to NGF injection, only partially blocked NGF-induced DNA synthesis, and thymidine levels were 50% of levels observed with NGF alone. Propranolol given prior to simultaneous administration of ISO + NGF inhibited thymidine incorporation to approximately the same extent as that observed with the β-antagonist and either ISO or NGF.

Since NGF characteristically affects the sympathetic innervation, it was important to determine whether the growth induced in pancreas by NGF was accompanied by changes in level of the adrenergic neurotransmitter and in number of β-adrenoceptors (since it is these that appear to mediate growth responses). Moreover, since it is the β_1-adrenoceptor only that is involved in growth mediation, the distribution of β_1- and β_2-adrenoceptors in the NGF-treated rats was examined. Finally, comparison was made to EGF since this growth factor would not be expected to affect sympathetic development.

The data in Table I show norepinephrine concentrations of pancreas of rats given NGF or EGF for 8 days, with initial injection being given to rats 5 and 10 days of age. A 31% increase in NE of pancreas was effected by NGF given two times daily from Days 5 to 13. Rats 10 days of age at the time of initial NGF injection showed no statistically significant increase in [NE]. In contrast, EGF caused no increase in NE of pancreas of either age group. (In fact, it was less than controls in the 10-day age group.)

[^{3}H]DHA and [^{3}H]QNB binding of pancreas of rats given either NGF or EGF for 8 days (animals were either 5 or 10 days at the time of initial injection) was not changed from that of controls (Table I). Furthermore, the values for [^{3}H]DHA and [^{3}H]QNB binding of the pancreas of young rats was identical to that of adult rats (Table I). However, the proportion of β_1- and β_2-adrenoceptors of pancreas in young rats differed from that of adults (Table I and previous data (2)). The proportion of β_1- and β_2-adrenoceptors was not affected by NGF or EGF, and values for pancreas of treated rats were the same as those of controls (Table I).

Receptor density of pancreatic membranes of 13-day-old rats was not changed after 2 days of administration of NGF, or ISO, or NGF + ISO, or when propranolol was given prior to administration of NGF, and [^{3}H]DHA and [^{3}H]QNB binding of pancreatic membranes was the same as that of untreated rats (Table II). The proportion of β_1- and β_2-adrenoceptors was also the same in all of the rats.

Discussion

Present data show that NGF has a stimulatory effect on DNA synthesis in pancreas of very young rats that is nearly equal to that induced by large doses of the β-adrenergic agonist, ISO (8). However, while all of the ISO-induced response appears to be mediated through activation of β-adrenoceptors, only 50% of the NGF-induced synthesis appears so mediated. Thus, propranolol completely blocked ISO-induced [^{3}H]thymidine incorporation, whereas it blocked only 50% of NGF-induced [^{3}H]thymidine incorporation. The effect of NGF was more marked than that of EGF; only a

Table I. Norepinephrine Concentration and Distribution of β_1- and β_2-Adrenoceptors in the Pancreas of Young Rats Injected with NGF or EGF for 8 Days[a]

Treatment	No. of days	Age at beginning	Age at sacrifice	[NE] (ng/g)	Receptor Density (fmol/mg membrane protein)			
					[^{3}H]DHA binding			[^{3}H]QNB binding
					−Antagonist	+Atenolol	+Butoxamine	
None	0	5	13	148 ± 11	62 ± 0	45 ± 0	16 ± 0	29 ± 1
EGF	9	5	13	148 ± 1	61 ± 0	49 ± 1	18 ± 1	28 ± 1
NGF	8	5	13	194 ± 4[b]	61 ± 1	46 ± 1	15 ± 1	28 ± 1
None	0	10	18	381 ± 71	63 ± 1	48 ± 3	17 ± 1	30 ± 2
EGF	8	10	18	228 ± 26	61 ± 1	44 ± 3	18 ± 1	30 ± 1
NGF	8	10	18	442 ± 97	62 ± 0	47 ± 4	19 ± 2	29 ± 2
None	0	120	128	746 ± 36	62 ± 1	59 ± 2	5 ± 0	31 ± 0

[a] Values are mean ± SE. [^{3}H]DHA binding of membrane receptors was done in the absence of antagonists (−antagonist), or in the presence of the selective β_1 (+atenolol)- or β_2 (+butoxamine)-adrenergic antagonists. NGF or EGF was given intraperitoneally two times daily for 8 days in a dose of 10 ng/kg body wt. Number of rats in each group: 4–6.
[b] Statistical difference from control ($P < 0.05$).

Table II. Properties of Membranes from the Pancreas of 13-Day-Old Rats Injected with NGF, ISO, or IN[a]/NGF for 2 Days[b]

Treatment	No. of rats	Receptor density pancreas (fmol/mg membrane protein)	
		[^{3}H]DHA binding	[^{3}H]QNB binding
None	4	60 ± 0.6	29 ± 0.3
ISO	4	61 ± 0.4	29 ± 0.4
NGF	10	61 ± 0.7	30 ± 0.8
NGF + ISO	6	61 ± 0.9	30 ± 0.8
IN + NGF	4	60 ± 0.8	30 ± 1.3

[a] IN, propranolol.
[b] Values are mean ± SE. ISO (50 mg/kg body wt), NGF (10 ng/kg body wt) and IN (3 mg/kg body wt) were injected twice daily for 2 days.

very modest increase in pancreatic DNA synthesis is effected by the latter (1).

The response of the pancreas of the young rat to NGF as well as ISO is thus similar to the response of parotid and submandibular glands (1, 7, 8). However, the β-mediated component is greater in the parotid than in the pancreas; the submandibular, on the other hand, shows a β-mediated component (7) that is similar in magnitude to that exhibited by pancreas. The difference may be a reflection of the different numbers of β-adrenoceptors normally present in these organs (1). The difference cannot be attributed to altered numbers of functional β-receptors in the NGF-treated organs, however, since these do not change in either salivary gland (1, 7, 8) or pancreas under any of the conditions described. In these young rats, NGF also caused a 31% increase in [NE] of pancreas (compared with controls) but no increase in rats 18 days of age or older. The EGF, however, did not cause any increase in [NE]. These differences are expected since NGF affects sympathetic nerve growth specifically (10, 11) whereas EGF chiefly affects epidermal growth (1, 4).

The distribution of β_1- and β_2-adrenoceptors was not changed by either NGF or EGF, and there were approximately 30% β_1- and 70% β_2-adrenoceptors in pancreas of controls as well as those of NGF- or ISO-treated animals. Thus, while the development of the sympathetic nerve is accelerated by the NGF, the number and kind of β-adrenoceptors is not affected by the increase in neurotransmitter concentration in the nerve fibers. Thus, an independence of nerve fiber and receptor development is implied by these data. Cholinergic receptors were also unaltered, as anticipated.

The compensatory growth response (1, 7, 8) that may influence interpretation of data for the parotid gland may have significance in analysis of data on pancreas also. Thus, the more marked increase in thymidine levels observed in the pancreas of partially desalivated rats given both ISO and NGF is a significant additional response that may include such an effect if the compensatory response can be considered to be caused by a humoral factor resulting from desalivation (14). If, on the other hand, as suggested by denervation experiments, the compensatory response is related to a neurally mediated increase in functional load on the parotid gland following ablation of the SM-SL glands (15, 16), then a humoral factor would not be involved in a compensatory growth response. Since desalivation itself caused no increase in thymidine incorporation in pancreas, the possibility of a humoral factor appears to be remote. The additional increment with NGF + ISO in the partially desalivated rats remains incompletely delineated.

In general, ISO is without effect or has only a modest stimulatory effect on DNA synthesis of the pancreas or parotid (1, 8) of rats without submandibular glands. However, DNA synthesis in the pancreas in the partially desalivated rats given injections of NGF is as high as that of intact rats treated with NGF. Thus, effects of NGF and ISO on pancreas closely resemble

the effects of ISO and NGF on parotid (1, 8). The data show that in the partially desalivated rats, the combined administration of ISO + NGF caused an increase in thymidine incorporation that is about 35% higher than that induced by NGF + ISO in the intact rats, and the propranolol-induced inhibition of ISO + NGF in intact rats is 68% of that observed in the partially desalivated rats. Thus, there are not only non-β-mediated NGF effects on DNA synthesis in pancreas (as was also reported for parotid), but some other growth inducing effect becomes evident when the submandibular glands are absent.

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