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Neurotrophins and Neurotrophin Receptors mRNAs Expression in Pancreatic Islets and Insulinoma Cell Lines

Paolo Bonini[1], Daniela Pierucci[1], Simona Cicconi[1], Ottavia Porzio[1], Renato Lauro[1], Lionel NJL Marlier[1,2], Patrizia Borboni[1]

[1]Laboratory of Molecular Medicine, Department of Internal Medicine, University of Rome "Tor Vergata". Rome, Italy. [2]National Research Council (CNR), Institute of Neurobiology and Molecular Medicine (INeMM). Rome, Italy

ABSTRACT

Context It is worth noting that islets and betaTC6-F7 cells share a common pattern of expression of neurotrophins and neurotrophin receptors. Recently, several studies have hypothesized a role for nerve growth factor in pancreatic development and maturation, suggesting that nerve growth factor may be a survival factor for pancreatic beta-cells.

Objective The aim of the present study was to investigate the pattern of expression of neurotrophins and their relative receptors both in rat pancreatic islets and in a wide panel of insulinoma cell lines.

Main outcome measures A semi-quantitative reverse-transcription polymerase chain reaction analysis was performed on ribonucleic acids extracted from these cell.

Results Reverse transcription-polymerase chain reaction analysis demonstrates that brain-derived neurotrophic factor, as well as neurotrophins 3 and 4, are expressed both in islets and in all insulinoma cells, while nerve growth factor is expressed only in islets, betaTC6-F7 cells and, at a low level, in RIN 1046-38 cells. Receptors protein tyrosine kinase A and C are ubiquitously expressed both in islets and insulinoma cells. Tyrosine kinase B is absent in HIT-T15 cells.

Conclusions These data indicate that betaTC6-F7 cells are a suitable model for studying the role of neurotrophins in the survival of beta-cells.

INTRODUCTION

It has recently been demonstrated that pancreatic beta-cells express functional receptors for nerve growth factor (NGF) and that NGF exerts some effects on beta-cells such as the induction of neuron-like differentiation, the stimulation of sodium current and the enhancement of early responsive gene expression (i.e. NGF-1A and c-fos) [1, 2, 3, 4, 5]. NGF exerts its biological effects on neuronal cells through specific cell surface receptors: $p75^{NTR}$ (p75), which is the low affinity NGF receptor whose function has not yet been completely elucidated, and $gp140^{Trk-A}$ (Trk-A, tyrosine-receptor kinase A), which is the high affinity NGF receptor which combines with $p75^{NGFR}$ to form a receptor complex with full biological activity [6]. The expression of both high- and low-affinity receptors for NGF

has been demonstrated in different insulinoma cell lines and in fetal rat islets, while the expression of Trk-A has been also demonstrated in adult islets [7, 8]. The cellular localization of Trk-A and NGF is developmentally regulated, suggesting that the neurotrophin system may play an important role in beta-cell development. Little is known about the expression and role of the other neurotrophins and their relative receptors in pancreatic beta cells [3, 5, 9].
The aim of the present study was to characterize the pattern of expression of neurotrophins and their receptors in pancreatic beta-cells and in pertinent insulinoma cell lines expression.

METHODS

Cells Cultures

Islet preparations were obtained from 6-month-old mice. The pancreases were excised from 3 mice and digested as previously described [10]. Subsequently the islets were handpicked under a stereomicroscope. Cells, free of exocrine tissue, were cultured in Rosweli Park Memorial Institute (RPMI) 1640 medium (Gibco-BRL, Gaithersburg, MD, USA) as previously described [10].
BetaTC6-F7 cells (kindly provided by Dr. Shimon Efrat, Tel Aviv University, Israel) obtained from transgenic mice expressing SV40 large-T antigen under control of the insulin promoter, were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco-BRL, Gaithersburg, MD, USA) supplemented with 15% horse serum (HS), 5% fetal calf serum (FCS) and 2 mM glutamine and used at passages 35-50 where physiological glucose responsiveness is maintained [11]. RIN 1046-38 cells were cultured in M199 medium (Gibco-BRL, Gaithersburg, MD, USA) supplemented with 10% FCS and 2mM glutamine. RIN cells were used at passages 19-25 in which glucose responsiveness is maintained [12]. INS-1 cells (passages 85-92) were cultured in RPMI 1640 medium, 1 mM sodium pyruvate, 50 μM beta-mercaptoethanol, 10% FCS and 5% HS. HIT-T15 cells (passages 72-82), a clonal hamster cell line, were cultured in RPMI 1640 medium supplemented with 10% FCS and 2.5 μg amphotericin B.
All the culture media contained 11.1 mmol glucose, 50 IU penicillin and 50 μg/mL streptomycin. Cells were subcultured once a week and maintained at 37 °C in an atmosphere of 95% humidified air and 5% CO_2.

RNA Preparation and RT-PCR Analysis

RNA was extracted by the guanidium thiocyanate method [13] and used for reverse transcription–polymerase chain reaction analysis (RT-PCR). Briefly, 1 μg RNA was reverse transcribed for 1 hour at 42 °C using 200 U Moloney murine leukemia virus (MMLV) reverse transcriptase (Gibco-BRL, Gaithersburg, MD) in the presence of 2.5 μM random hexamers and 200 μM nucleotides (Amersham Pharmacia Biotech, Cologno Monzese, MI, Italy) in 20 μL final volume. Successively, 2 μL of each cDNA were PCR amplified using 2.5 U Platinum Taq except for $p75^{NTR}$ for which 2.5 U Hot Start (Qiagen SpA, Milan, Italy), was used in the presence of 15 pmol of specific primers. To normalize cDNA amounts used during the PCR amplification, a parallel amplification was performed using glyceraldehyde-3-phosphate dehydrogenases (GAPDH) specific primers (not shown).
After a 5 min denaturation step, PCR cycles consisted of 30 sec denaturation, 30 sec annealing and 1 min extension (5 min final extension) were performed using a Perkin Elmer 2400 thermal cycler (Perkin Elmer Corporation, Norwalk, CN, USA). The number of cycles depended on the relative abundance of each target analyzed and is indicated below. Primer sequences were as follows: NGF forward 5'-AAGGACGCAGCTTTCTATAC and reverse 5'-TGTGGAAGACTGGGTGGGT

(60 cycles); brain-derived neurotrophic factor (BDNF) forward 5'-ATGGGACTCTGGAGAGCGTGAA and reverse 5'-CGCCAGCCAATTCTCTTTTTGC (50 cycles); neurotrophins 3 (NT3) forward 5'-CTTATCTCCGTGGCATCCAAGG and reverse 5'-TCTGAAGTCAGTGCTCGGACGT (55 cycles); neurotrophins 4 (NT4) forward 5'-TTCTGGCTCCTGAGTGGAC and reverse 5'-AGTCAACGCCCG-CACATAG (50 cycles); Trk-A forward 5'-GTGCTCAATGAGACCAGC-TTC and reverse 5'-CTTCAGTGCCCTTGACAGCCAC (50 cycles); Trk-B^+ forward 5'-TACTGGGACGTTGGGAATTTGG and reverse 5'-CCCTCTTCAGAACGATGTTGTG (45 cycles); Trk-B^- forward 5'-TACTGGGACGT-TGGGAATTTGG and reverse 5'-CCTTTATCTCAGCTACCCATCC (60 cycles); Trk-C forward 5'-TGGACTGGATAGTCACTGG and reverse 5'-TGGGTCACAGTGATAGGAG (45 cycles); p75 forward 5'-GAGCCACCAGAGCGTGTG and reverse 5'-GGGGATGTGGCAGTGGAC (60 cycles). The number of cycles indicated were used in pilot experiments to allow PCR amplification in the linear range.

ETHICS

Animals used for islet preparations received humane care according to the standard criteria outlined in the "Guide for the Care and Use of Laboratory Animals" as prepared by the National Academy of Sciences.

STATISTICS

No statistical evaluation of the data was performed due to the qualitative nature of this study.

RESULTS

RT-PCR analysis demonstrated a cell-specific pattern of neurotrophin/neurotrophin receptor expression (Figure 1). In particular, NGF mRNA expression is virtually limited to islets and betaTC6-F7 cells. It is barely detectable in RIN and undetectable in the other cell lines.

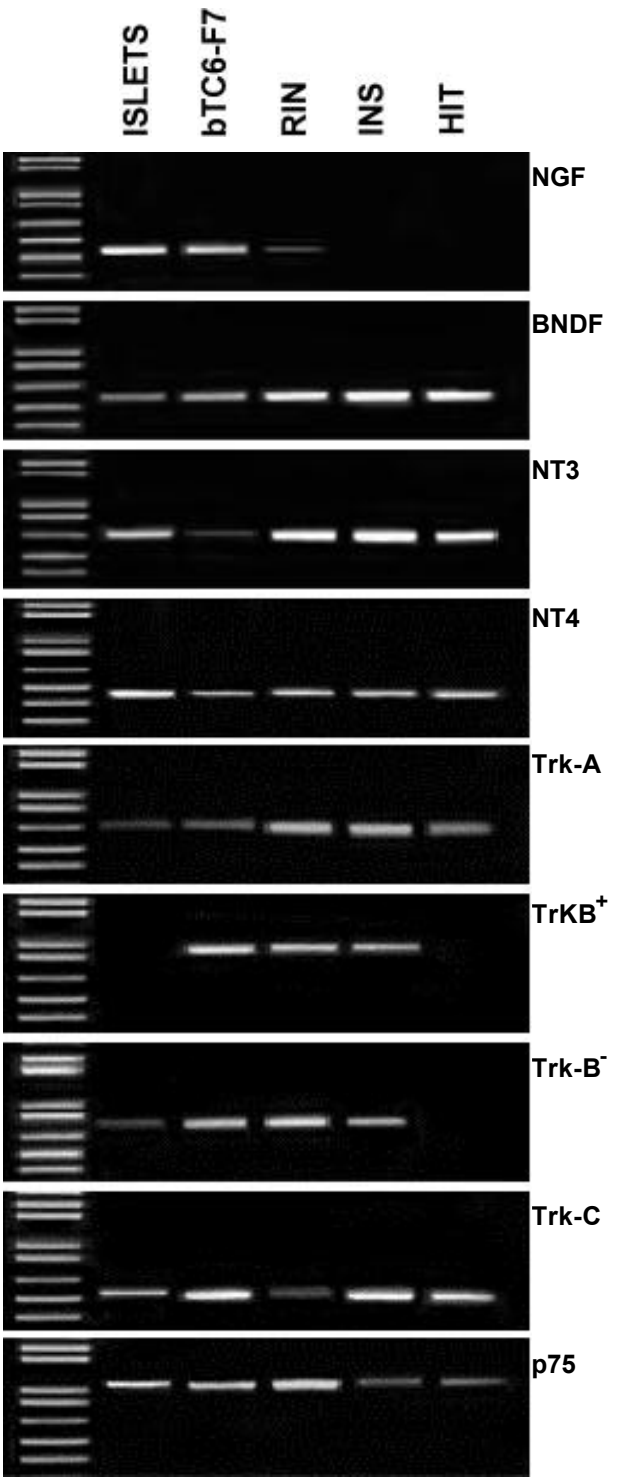

Figure 1. RT-PCR analysis. The marker on the left is a $1Kb^+$ ladder (Gibco).

BDNF and NT4 mRNA expression is present in all groups. NT3 mRNA is expressed in all groups but is barely detectable in betaTC6-F7 cells.
Concerning the pattern of neurotrophin receptor expression, Trk-A mRNA is uniformly expressed, although less so in islets and betaTC6-F7 cells. Trk-B^+ mRNA is not expressed in islets and in HIT-T15 cells while Trk-B^- mRNA is absent in HIT-T15 cells and barely detectable in islets; TrkC mRNA is expressed in all groups, although to a lesser degree represented in RIN cells. Finally, p75 mRNA is expressed in islets and in all the cell lines, and is less abundant in RIN and HIT cells.
Although this was not a quantitative study, the amount of RNA from each cell line used for RT-PCR was normalized. Therefore the amplitude of bands and the number of PCR cycles applied to get uniform bands for a given target are consistent with differences in the mRNA contents. In particular, NGF mRNA required 60 cycles while BDNF or NT4 required only 50 cycles to obtain a comparable signal. Similarly, p75 was clearly detectable after 40 cycles, while the kinase *minus* isoform of Trk-B required 60 cycles.

DISCUSSION

The family of neurotrophic factors includes NGF, the first neurotrophin identified, BDNF, NT3 and NT4. Signal transduction by these neurotrophins is initiated by binding to specific high affinity tyrosine-kinase receptors, called Trk-A for NGF and NT3, Trk-B for BDNF and Trk-C for NT4. All the neurotrophins interact with the low affinity receptor p75, whose role in the neurotrophin signal transduction is not completely elucidated [14].
NGF and BDNF are expressed in a limited number of peripheral tissues [15]; in particular NGF has been detected in lymphocytes and pancreatic beta–cells [3, 16], while BDNF has been identified in dorsal root ganglia [17]. On the contrary, NT3 is almost omnipresent being localized in the heart, kidney, gut, lung, spleen, liver, muscle, skin, secretory cells of the submandibular gland and epithelial cells of secondary and tertiary follicles in the ovary [18].
Neurotrophin 4/5 (NT4/5) is a member of the neurotrophin family known to influence survival and to have other effects on a variety of neuronal cells. Although NT4/5 mRNA has been found in various effector tissues of the rat and human, the concentration of NT4/5 protein in tissues has not been previously reported due to the lack of a suitable methodology. A recently developed quantitative two-site enzyme-linked immunosorbent assay for the estimation of NT4/5 in pre- and postnatal rat tissues showed that NT4/5 is present in most embryonic tissues but was rarely detectable in postnatal tissues, with the notable exception of the testis [19].
Previous studies have demonstrated the expression of high- and low-affinity receptors for NGF in pancreatic islets and in various beta-cell lines as well as in numerous non-neuronal tissues by both Northern blot analysis and binding studies [1, 3, 4, 9, 20]. Trk-B receptor has been found only in truncated forms in non-neural tissues such as the spleen, submandibular gland, testes, kidney and pituitary gland [21]. On the contrary, Trk-C has been found in the thymus, lung, kidney, stomach and testes with discordant data regarding findings of truncated or full-length receptor forms. Recently the presence of full-length Trk-C mRNA and protein has been demonstrated in INS-1 cells, where NT3 determines an increase in intracellular free calcium and is not followed by changes in insulin secretion [22].
The present study points out that in islets the pattern of neurotrophin/neurotrophin receptor expression includes all the neurotrophins with Trk-A and C mRNAs. On the contrary, in the beta-cell lines, NGF mRNA expression is lacking except in betaTC6-F7 cells. In RIN

cells it is expressed at a very low level. Trk-A and C are omnipresent, even though less abundant in RIN cells, while Trk-B is lacking in HIT cells. Finally, islets express the low affinity receptor for NGF as well as betaTC6-F7 cells and RIN cells, while it is less abundant in INS and HIT cells.

These data suggest that islets and betaTC6-F7 cells share a common pattern of neurotrophin expression which is unusual as compared to the other tested cell lines tested due to the presence of NGF. Similarly, islets share a common pattern of expression with INS-1 and HIT cells relative to the NT3/TrkC axis.

We hypothesize that NGF and NT3 can act at the level of pancreatic beta−cells by independent autocrine loops which can be relevant for beta-cell functioning and we suggest that betaTC6-F7 cells represent a unique model for studies on the NGF mechanism of action. In addition, INS-1 and HIT-T15 cells are useful tools for studies of the mechanism of action of NT3.

Considerable evidence has recently been provided indicating a role for NGF in pancreas development and maturation. In fact, it has been demonstrated that inhibition of the tyrosine-kinase activity of the NGF receptors causes an impairment of islet morphogenesis [4]; NGF receptor expression in beta-cells is regulated by prolactin and/or the growth hormone, two hormones which are involved in pancreas development and beta−cell function [23]. Furthermore, NGF has been demonstrated to induce neuron-like differentiation in beta-cells [5, 9, 24]. Finally, NGF increases $gp140^{Trk-A}$ expression in islets, suggesting that the NGF autocrine effects are mediated through activation of $gp140^{Trk-A}$ [2]. The role of $p75^{NTR}$ in beta-cells has not yet been clarified. The NGF low affinity receptor is considered to be involved in the modulation of apoptosis/survival in neurons which is consistent with its structural similarity to Fas/Apo-1, tumor necrosis factor receptors I and II and CD40 [25, 26, 27] . Based on these data, it can be hypothesized that NGF plays a role in the modulation of apoptosis/survival of islet beta-cells. Beta cell susceptibility to apoptosis influences the occurrence of diabetes by reducing the beta-cell mass [28, 29] . Therefore, the understanding of the mechanisms influencing this highly regulated process is extremely important.

In conclusion, we have characterized the neurotrophin/neurotrophin receptor pattern of expression in a panel of beta−cells, providing basic information as to the possible role of neurotrophic factors in the regulation of beta−cell apoptosis/survival. Furthermore, we have determined the cellular models useful for studies in this field.

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Key words Insulinoma; Islets of Langerhans; Nerve Growth Factor; Polymerase Chain Reaction

Abbreviations BDNF: brain-derived neurotrophic factor; DMEM: Dulbecco's modified Eagle's medium; FCS: fetal calf serum; GAPDH: glyceraldehyde-3-phosphate dehydrogenases; HS: horse serum; MMLV: Moloney murine leukemia virus; NT: neurotrophins; RPMI: Rosweli Park Memorial Institute; Trk: tyrosine-receptor kinase

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Correspondence
Patrizia Borboni
Laboratory of Molecular Medicine
Department of Internal Medicine
University of Rome "Tor Vergata"
Via di Tor Vergata, 125
00136 Rome

Italy
Phone: +39-06-7259.6530
Fax: +39-06-7259.6538
E-mail address: borboni@uniroma2.it

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