Short Communication

Transfection of 6-23 a rat thyroid carcinoma cell line, with the Neuropeptide Y cDNA

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Abstract
Neuropeptide Y (NPY) is a 36 amino acid peptide found throughout the central and peripheral nervous system of rat and human. NPY has been proposed to play an important role in satiety. The aim of this study was to produce cell lines that secrete high levels of bioactive NPY. For this purpose, the complementary DNA (cDNA) that encodes NPY was isolated by PCR. The cDNA was then cloned into pCEP4, to form pCEP4NPY. 6-23 cells were transfected with pCEP4NPY by electroporation. Transfected cells were selected by the addition of hygromycin B to the culture medium. Resistant colonies were picked and transferred to 96-well plates. The medium was tested for IR-NPY using a specific NPY radioimmunoassay (RIA). The IR-NPY secreted by the cells was characterized by sephadex G_{50} chromatography and reversed phase fast protein liquid chromatography (FPLC). It was found to co-elute with the synthetic standard in both cases. RNA was extracted from the cells and subjected to Northern blot analysis using labeled NPY cDNA as a probe. The cells were found to express high levels of NPY at mRNA levels.
Keywords*: NPY; cDNA; Transfection; Cell line.*

Neuropeptide Y (NPY) is a 36 amino acid peptide that was first isolated from porcine brain by Tatemoto *et al*. (1982).

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NPY-immunoreactivity is widespread throughout the central nervous system (CNS) of the rat and human (Polak and Bloom, 1984). In the periphery, sympathetic neuropeptide Y plays a role as a vasopressor and vasoconstrictor. NPY is a vasoconstrictor in many arteries *in vitro* and *in vivo*, and several mechanisms of action have been proposed (Fredholm *et al*., 1985). NPY-induced feeding is potently inhibited by amylin, a peptide co-released with insulin from the pancreatic beta cells (Morris and Nguyent, 2001).

NPY has a number of effects including regulation of appetite, sexual behavior, and reproductive function. The most significant effect of NPY administration into the CNS is a dramatic and sustained increase of food intake, first observed by Clark *et al*. (1984).

Neuropeptie Y can interfere with other hormones such as galanin, ghrelin, leptin, PYY, PP, and amylin. It has been reported that following central injection of leptin, NPY mRNA expression in goldfish brain was reduced (Volkoff *et al.*, 2003). Pharmacological evidence indicated that ghrelin (a stomach-derived orexigenic hormone) effects on food intake are mediated by NPY and agouti-related protein (AGRP) in the CNS (de Ambrogi *et al*., 2003; Chen *et al*., 2004). Intracerebroventricular (ICV) injection of NPY, galanin and ghrelin, stimulated growth hormone releasing hormone (GHRH). In addition NPY suppressed large GH pulses (Mogi *et al.*, 2004).

Since NPY has several effects in energy metabolism, cardiovascular system, respiratory system, thy-

roid functions and sexual hormones when administer peripherally or centrally, therefore the present study was conducted to prepare a condition for the chronic study of the effects of NPY. To study the chronic effects of this peptide, the target tissue should be exposed to this hormone constantly and continuously. Although an infusion system such as Alzet pump can be used to deliver the peptide but the problem is that this pump should continuously be filled by fresh solutions of the peptide because the peptide has a short half-life and rapidly inactivated in solution.

The aim of this project was to prepare an immortal cell line that constantly and continuously overexpressed and secreted NPY. Therefore 6-23 (a rat thyroid carcinoma cell line) was transfected with NPY cDNA to produce bioactive NPY. This transfected cell line was then used to study the chronic effects of NPY (Mahmoodi *et al*., 2004).

Production of NPY expression vector: Total RNA was extracted and purified from rat hypothalamus, using phenol-chloroform extraction method (Chomczynski and Sacchi, 1987). Full length of NPY cDNA was obtained by polymerase chain reaction (PCR) and the PCR product (NPY cDNA) was sequenced by Sanger method (Sanger *et al*., 1977) and the sequence of NPY cDNA was identical to published sequence. The plasmid vector, pCEP4 (Invitrogen, Leek, Netherland) was used to clone the NPY cDNA. This vector carries the gene for hygromycin-B and ampicillin resistance for selection of recombinants in mammalian and bacterial cells respectively. The clones contain NPY cDNA (pCEP4-NPY) was purified by agarose gel electrophoresis and Cesium chloride gradient centrifugation (Sambrook *et al*., 1989) and stored in -20°C until used.

Generation of stable cell line over expressing NPY

Cell culture: To generate a stable cell line that secreted NPY, the 6-23 clone 6, (ECACC, Porton Down, Wiltshire, UK) a rat modularly thyroid carcinoma cell line was used. The cells were adherent and grew as a monolayer which was routinely cultured in 80 cm^2 flasks (Nunc Products, Gibco-BRL), in Dulbecco s Modified Eagle Medium (DMEM) containing 4.5 mg/ml glucose, with 10% (V/V) heat inactivated horse serum, 5% heat inactivated foetal bovine serum (ΔFBS), 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C in 6% CO_2 atmosphere. The medium was changed every 2-3 days and the cells sub-cultured when 70-80% confluent using trypsin/EDTA.

Transfection of cell line with pCEP4-NPY: 6-23 clone 6 proved resistance to calcium phosphate mediated transfection (Sambrook *et al*., 1989), therefore electroporation was used to transfect this cell line as described by us previously (Mahmoodi *et al*., 2004). After transfection, the medium was exposed to the cells overnight and assayed for NPY immunoreactivity (IR-NPY), using specific radioimmunoassay (RIA) as explained previously (Mahmoodi *et al*., 2004). Colonies from 96-well plates secreting IR-NPY were expanded into 12-well plates, then 25 and 80 cm^2 and finally into 175 cm^2 flasks for continuous stock, RNA extraction and RIA.

Sephadex G50 column chromatography and reversed-phase fast protein liquid chromatography (RP-FPLC): Conditioned medium from 12-well plates and 80 cm^2 flasks was either loaded onto sephadex G50 column or injected into FPLC column. The fractions of both columns were collected and assayed for IR-NPY as previously described by us (Mahmoodi *et al*., 2004). Analysis of conditioned medium from 6-23-NPY cells by size exclusion chromatography and RP-FPLC gave a major peak of immunoreactivity eluting at the same position as the synthetic standard (Fig. 1A and 1B).

Secretion studies: Cells grown to 80% confluence were subcultured and plated at a density of 2×10^5 cells per well in 24-well plates and grown to 70-80% confluence. After this time, the medium was removed and the cells washed with FBS free medium. Fresh medium (1 ml) containing FBS was added to each well and the cells incubated at 37°C and 6% CO_2. Medium was removed from 8 separate wells at the following time points: 0, 0.25, 0.5, 0.75, 1, 1.5, 2, 3, 4, 5, 8, 10, 18, 24, 30, 36, 48, 56 and 72h and NPY concentration was measured by RIA. Levels of NPY in medium conditioned by cells rose up to 24h then decreased. The rate of NPY secretion by the cells was 315.4 f mol/ml conditioned medium/hour (Fig. 2).

RNA extraction and Northern blot analysis: Total RNA was extracted from transfected and untransfected (control) cells by phenol-chloroform extraction (Chomczynski and Sacchi, 1987).

Northern blot analysis was used to analyze the amount of RNA expression in cells as described previously (Mahmoodi *et al*., 2004). When RNA extracted from transfected cells were analyzed by northern blotting, they were found to express high levels of NPY mRNA, whilst RNA extracted from the untransfected (control) cells was negative (Fig. 3).

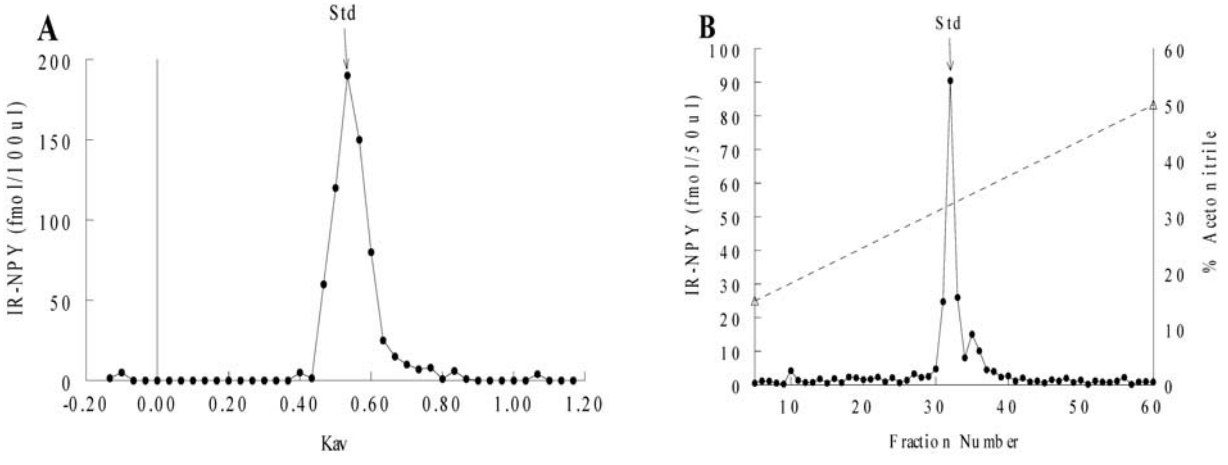

Figure 1. A: Sephadex G_{50} size exclusion Chromatogram of NPY-IR in conditioned medium from 6-23-NPY cells**;** **B**: Reversed phase FPLC Chromatogram of NPY-IR in conditioned medium from 6-23-NPY cells.

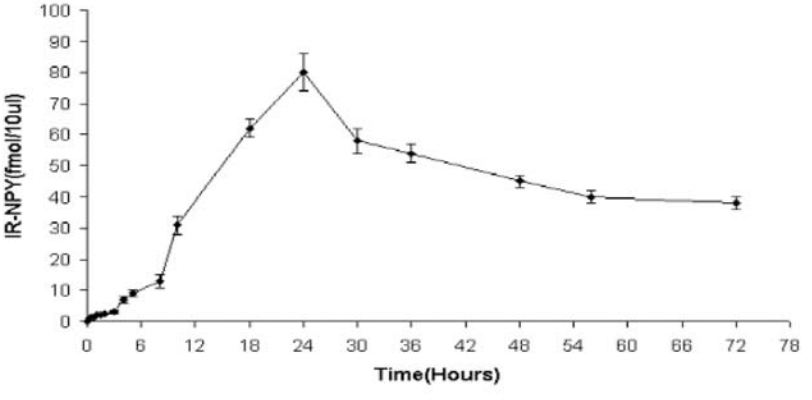

Figure 2. Time course of secretion of NPY by 6-23-NPY cells. Data represents mean± SEM, n=8.

The aim of this project was to prepare a cell line that over-expressed and secreted NPY, to study the chronic effects of this peptide in various tissues. Other investigators have found that NPY is unstable when stored in mini-osmotic pumps and retains its bioactivity for a short time. To overcome this problem, a novel approach was needed that would allow the continuous infusion of newly synthesized NPY. The rational behind production of a cell line that over express NPY was based on the experiments of Hammer *et al*. (1992). They had shown that it was possible to study the effects of peptide hormones on pituitary function by ectopic production in the periphery (Hammer *et al*., 1992). The production of a plasmid that when transfected into mammalian cells will drive expression of NPY was a straightforward task. Following the isolation and cloning the NPY into pCEP4, the next stage was to select suitable cell lines for the over expression of NPY. The requirements for the cell lines were that they had to be eukaryotic and capable of processing and releasing peptide hormones and are derived from easily available strains of animal. 6-23 clone 6 was chosen in this project that is derived from endocrine tissue and is known to secrete other peptide hormones. This cell line has been used to produce other bioactive neuropeptides such as galanin and neuromedin U

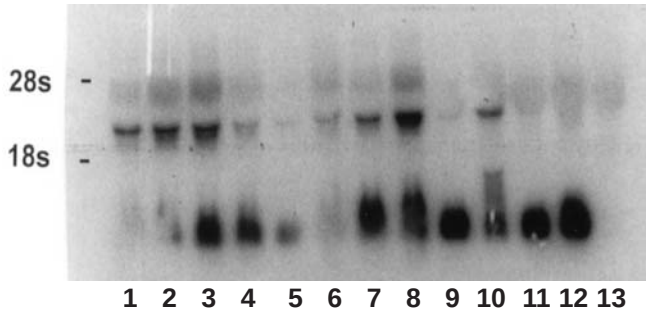

Figure 3. Northern blot analysis of 50 µg of total RNA extracted from 6-23 cells transfected with pCEP4-NPY, 1-12 individual clones, 13 untransfected 6-23 cells. 28S and 18S mark the position of the 28S ribosomal RNA and 18S ribosomal RNA respectively.

(NMU) and shown to secrete a high level of these two hormones (Austin 1996, Gardiner, 1998). Therefore this cell line was chosen as a suitable candidate for synthesis and processing of bioactive NPY, indeed bioactive NPY was produced by this cell line when transfected with pCEP4-NPY.

6-23 clone 6 cell line has been successfully transfected with the full length of NPY. High levels of expression of the corresponding mRNA have been shown using northern blot analysis. Detection high levels of NPY by radioimmunoassay suggested correct translation of the transcripts.

The other part of this project showed that the NPY secretion pattern is vary by the time course of incubating cells in culture medium. The level of NPY in medium conditioned by transfected cells initially rose up to 24h and then decreased. This decrease in NPY concentration is probably due to degradation of the secreted peptide, with other secreted factors feeding back on the cells to prevent further release of NPY. Support for this hypothesis comes from the observation that replacement of this media after 24h with fresh medium resulted in the same secretion time course. Degradation may be via a secreted protease (Yap and McGee 1991).

Overall this data has shown that the 6-23 clone 6 cell line was successfully transfected with NPY cDNA cloned into pCEP4 plasmid and different procedures were shown that transfected cells were able to express and secrete NPY. Therefore this transfected cell line can be used to demonstrate the chronic effects of NPY in future works.

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