Secretion of Bax-Like Protein into Systemic Circulation from the Posterior Pituitary in the Rat Hypothalamo-Posterior Pituitary System

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To investigate the possibility of the neurosecretion of Bax-like protein in the adult rat, we examined its distribution in the hypothalamo-posterior pituitary system using light and electron microscopic immunohistochemistry. We also measured the serum Bax-like protein using an enzyme immunoassay. Neurons in the paraventricular nucleus (PVN) and supraoptic nucleus of the hypothalamus exhibited intense Bax-like immunoreactivity (Bax-IR). Bax-like immunoreactive axons projected to the posterior pituitary through the internal layer of the median eminence. By immunoelectron microscopy, Bax-IR was observed in the granulated vesicles and in the membrane of the rough-surfaced endoplasmic reticulum in the neuronal perikarya in the medial magnocellular division of PVN. It was also detected in

the secretory vesicles in the nerve terminals of the posterior lobe of the pituitary gland. Double-labeling histochemistry revealed that almost all Bax-like immunoreactive substances were present in the oxytocin neurons, and many Bax-like immunoreactive neurons were nitro-oxidergic neurons. The enzyme immunoassay revealed that the average Bax-like protein concentration in the serum was 39.0 ± 9.6 pmol/ml in the adult male rat. Colchicine treatment significantly increased the densities of Bax-IR in the PVN and decreased the serum Bax-like protein levels. From these results, Bax-like protein is considered to be produced in the hypothalamic oxytocin neurons, transported through axoplasmic flow and secreted into systemic circulation at the posterior pituitary as a "neurohormone".

Key words: Bax, Neurosecretion, Immunohistochemistry, Hypothalamus, Rat

I. Introduction

The Bcl-2-associated X protein, Bax, was first identified as a biologically active, dominant regulator of apoptotic cell death [17]. Programmed cell death was thought to be related to the hetero-dimerizing ratio of Bcl-2/Bax [7, 25]. The proapoptotic members of the Bcl-2 family are considered to be self-producing in apoptotic cells because Bcl-2 family genes are induced within the apoptotic cell

itself [4, 11, 20]. Immunohistochemical investigations of adult mammals have revealed the widespread distribution of Bax immunoreactivity in several organs of the body such as hematolymphoid tissues [16], cardiovascular system, reproductive tissues [10]. Bax protein may participate in active cellular turnover with counteracting Bcl-2 protection against apoptotic cell death.

On the other hand, intense Bax-like immunoreactivity (Bax-IR) is consistently located in the neuronal perikarya and fibers of neurosecretory hypothalamic nuclei [23]. These hypothalamic neurons do not display neuronal degeneration. The above findings suggest that Bax-like protein in the hypothalamic neurons is secreted into systemic circulation, and may maintain homeostatic regulation in the peripheral tissues in addition to its role concerning neuronal apoptosis. In the present study, we investigated

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the neurosecretion of Bax-like protein by immunohistochemistry of the rat hypothalamus and pituitary gland, and by enzyme immunoassay (EIA) using rat serum.

II. Materials and Methods

Before each experimental procedure, all animals (48 adult male Sprague-Dawley rats weighing 250–350 g; SLC Co., Shizuoka, Japan), were deeply anesthetized with sodium pentobarbital (70 mg/kg body weight, i.p.).

Immunohistochemistry

Rabbit anti-serum Bax (P-19) (sc-526, Santa Cruz Biotechnology, Santa Cruz, CA) immunohistochemically detected Bax-IR. This antiserum is an affinity-purified

polyclonal antibody raised against a peptide corresponding to amino acids 43–61 of Bax- α protein of mouse origin. Immunohistochemical specificity was confirmed by the immunoabsorption test which resulted in the disappearance of immunohistochemical staining, by adding 10 μ g/ml of homologous peptide (sc-526 P, Santa Cruz) against the antibody to the working solution. Anesthetized animals were transcardially perfused with phosphate buffered saline (PBS), followed by a fixative containing 4% formaldehyde in 0.1 M phosphate buffer (PB) at pH 7.4. Brains were postfixed for 24 hr, and cryoprotected in 20% sucrose in 0.1 M PB. Frozen coronal sections (25 μ m in thickness) were incubated in anti-Bax antiserum (dilution 1 : 5000) for 24 hr at 4°C, then processed as previously described [23]. Finally sections were exposed to the

Fig. 1. Light microscopic immunohistochemistry shows Bax-like immunoreactive neuronal cell bodies and fibers in the PVN (a) and the SON (b). Immunoreactive neuronal fibers containing Herring bodies are distributed in the internal layer of median eminence (c). Many Bax-like immunoreactive axon terminals are located in the posterior pituitary lobe, but not in the intermediate lobe (d). V, third ventricle; oc, optic chiasm; P, posterior lobe; I, intermediate lobe. All scale bars = 100 μ m.

3,3'-diaminobenzidine (DAB) solution and Bax-IR was visualized as brown products.

Electron microscopy

The rats were transcardially perfused with a fixative containing 0.35% glutaraldehyde and 4% formaldehyde in 0.1 M PB, and the brains were postfixed for 24 hr. Serial frontal sections of hypothalamic regions (30 μ m in thickness) were processed for immunocytochemistry with anti-Bax antisera (dilution at 1 : 2000). Immunostained sections were postfixed by a chilled 1% OsO₄ solution, dehydrated and embedded in an Epon 812 mixture (Nacalai Tesque Inc., Kyoto, Japan). Ultra-thin sections stained with uranyl acetate (Merck, Darmstadt, Germany) and lead citrate (Nacalai Tesque Inc.) were examined by an electron microscope (JEM 200 CX).

Western immunoblotting

The hypothalami including paraventricular nucleus (PVN), anterior and posterior pituitary, were dissected from 10 rats as described [10], then the protein content

was determined by the Bradford method [3]. The protein samples (50 μ g) were separated by SDS-PAGE (14% acrylamide), and transferred to a polyvinylidene difluoride membrane (Micron Separations, Inc., Westboro, MA), that was then soaked in Bax-antiserum (1 : 1000), followed by Immuno-Star™ Chemiluminescent Protein Detection Systems (170-5011, Bio-Rad Laboratories, Hercules, CA). The membrane was exposed to X-ray film (Hyperfilm ECL™, Amersham, Buckinghamshire, England), then developed and scanned with a laser scanner.

Double labeling histochemistry

Colocalization of Bax-like protein with oxytocin (OXY), arginine vasopressin (AVP) and nitric oxide synthase (NOS) were investigated in hypothalamic neurons by double labeling histochemistry. Frozen sections (20 μ m in thickness) containing PVN and supraoptic nucleus (SON) were made. DAB-immunohistochemistry was first performed on the sections with anti-Bax serum (1 : 5000). Thereafter, we performed *in situ* hybridization histochemistry for OXYmRNA [8, 21, 24]. Oligonucleotide probe

Fig. 2. Schematic drawing of Bax-like immunoreactive neurons distributed in major divisions of the SON and the PVN. Scale bar=1 mm. (A; Bregma -1.4 mm, B; -1.6 mm, C; -1.8 mm, D; -2.0 mm) AC, anterior commissural nucleus; Cir, nucleus circularis; f, fornix; MFB, medial forebrain bundle nuclei; opt, optic tract; oc, optic chiasm; V, third ventricle; PaDC, paraventricular nucleus, dorsal medial cap; PaLM, paraventricular nucleus, lateral magnocellular division; PaMM, paraventricular nucleus, medial magnocellular division; PaPo, paraventricular nucleus, posterior division; PaV, paraventricular nucleus, ventral division; PeM, magnocellular periventricular nucleus.

complementary to the rat OXY was synthesized by an automated synthesizer (coding amino acid 117–124; 5'-CTCGGAGAAGGCAGACTCAGGGTCG-3'). The 3' end of these oligonucleotides were labeled with 50 pmol [³⁵S]-dATP (6000 Ci/mM; NEN Life Science, Boston, MA). After *in situ* hybridization, the sections were visualized by autoradiography.

The colocalization of Bax-like protein and AVP was examined by double-labeled immunofluorescence histochemistry. The sections were incubated in a mixture of rabbit anti-Bax (1:5000) and guinea pig anti-AVP (1:10000, Peninsula Lab., Belmont) primary antibodies for 3 days at 4°C. Subsequently, the sections were incubated in a mixture of goat anti-guinea pig fluorescein

Fig. 3. Electron microscopic features of Bax-like immunoreactive neurons in the PVN. Round nucleus with no degeneration (a). High-magnification of (a) shows granulated vesicles in neuronal perikaryon (arrows) and Bax-IR in the membrane of rER (arrow heads) (b). Axon terminal containing many immunoreactive transporting vesicles adjoined to extracellular pericapillary space (arrow) (c). (d) High magnification of (c). N, nucleus; M, mitochondria. Scale bars=5(a), 2(b) 1(c) and 0.5 μ m (d)

isothiocyanate (FITC, Bethyl, 1 : 2000) and donkey anti-rabbit rhodamine (TRITC, Chemicon, 1 : 1000).

To examine the colocalization of Bax-like protein and NOS, we performed NADPH-diaphorase histochemistry combined with Bax-like immunohistochemistry. Since NADPH-diaphorase positive and NOS immunoreactive neurons are identical in the hypothalamic nuclei, NADPH-diaphorase histochemistry was performed to detect NOS immunoreactive neurons as described [14], and immunohistochemistry to detect Bax-IR was then performed using anti-Bax (1 : 5000).

Colchicine-injection

We examined the transport of Bax-like protein through axoplasmic flow [2, 18]. Colchicine (Sigma) was injected (60 μ g/10 μ l saline) into the left lateral ventricle in twelve animals (four for immunohistochemistry and eight for EIA) 48 hr prior to sacrifice. Bax-like immunoreactive levels in the neuronal perikarya of the hypothalamic PVN were quantified in control rats (n=4) and those given colchicine (n=4). Serial cryostat sections (25 μ m in thickness) were processed for immunohistochemistry. About 10 sections per rat included the whole magnocellular paraventricular nucleus (MaPVN), and every second section (total 5 sections) was analyzed. Images were captured using a grayscale CCD digitizing camera system and immunoreactivity was quantified on NIH images using a computer system. Ten neurons were randomly selected from each section and the average grayscale intensity of the area of interest neuronal perikarya was determined.

EIA of serum Bax-like protein

Microtiterplates (Nunc, Roskilde, Denmark) were coated with 0.5 μ g of sheep-anti-rabbit IgG(Fc) (ICN, Costa Mesa, CA) per well dissolved in 100 μ l of coating buffer (10 mM PBS). The biotinyl partial Bax peptide (25 μ l), anti-Bax serum (100 μ l), and 25 μ l of rat serum sample or standard Bax peptide (range, 200 to 0.048 ng/ml) were simultaneously placed into wells with assay buffer, and incubated for 1 hr with 20 ng streptavidin-peroxidase (Oncogene, Cambridge, MA). The reaction was measured at 450 nm using a microplate reader (Multiscan MS, Dainippon Pharmaceutical, Osaka, Japan). Welch's test statistically compared the Bax-like protein levels between colchicine-treated and non-treated rats.

III. Results

Immunohistochemistry

The GenBank database (accession #U49729), revealed no significant homology of Bax with other known genes and protein sequences. Large numbers of the intensely Bax-like immunoreactive neurons were distributed in the MaPVN and SON (Fig. 1a, b). In these neurons, intense immunoreactivity was observed in the cytoplasm and nerve fibers. Immunoreactive neuronal processes including Herring bodies were also observed in the internal layer of the median eminence (ME) (Fig. 1c). Many Bax-like immunoreactive axon terminals were located in the posterior pituitary (Fig. 1d), but not in the anterior pituitary (data not shown). Neurons with Bax-IR in the PVN and SON were presented in the schematic drawing (Fig. 2). Neurons in the paraventricular dorsomedial cap and posterior subnucleus of the PVN are projecting to the brain stem and spinal cord, and related to the autonomic function [1]. Those non-endocrine projection neurons in the PVN and neurons of the dorsal part in the SON also contained Bax-IR (Fig. 2).

Electron microscopy

Immunoelectron microscopy revealed immunoreactivity in the membranes of rough-surfaced endoplasmic reticulum (rER), dense granulated vesicles in the cytoplasm, and in neuronal process in the MaPVN (Fig. 3a, b). In the internal layer of ME, nerve processes containing immunoreactive vesicles were intermingled with non-immunoreactive nerve processes (data not shown). In the posterior pituitary, immunoreactive and non-immunoreactive axon terminals were intermingled. Some axon terminals containing immunoreactive secretory vesicles adjoined the pericapillary space (Fig. 3c, d).

Western immunoblotting

Western blotting revealed a single band in the PVN, SON, and posterior pituitary, corresponding to a molecular mass of 21 kDa, but not in the anterior pituitary (Fig. 4).

Fig. 4. Immunoblot analysis of Bax-like protein in rat hypothalamo-posterior pituitary system. Positions of molecular weight markers are indicated in kilodaltons (kDa). (lane1, tissues in PVN) (lane2, SON) (lane3, posterior pituitary) (lane4, anterior pituitary)

Fig. 5

Fig. 6. (Left): Computer assisted analysis of Bax-like immunoreactive density (average intensity value per pixel, maximum intensity, 256). Perikaryonal immunoreactivities of PVN were significantly increased in colchicine treated ($n=4$), compared with non-treated control animals ($n=4$). Welch's test statistically compared the Bax-IR between the two groups. Values are expressed as means $\pm$ S.D. *, Significantly different from control at $P < 0.01$. (Right): Representative Bax-like immunoreactive neurons in the PVN of colchicine treated (a) and non-treated (b) animals. Intense immunoreactivity in neuronal perikarya and processes in the paraventricular nucleus of colchicine treated animals. All scale bars = 200 μ m. V, third ventricle.

Double labeling histochemistry

Immunohistochemistry with *in situ* hybridization for OXYmRNA revealed colocalized Bax-IR and OXYmRNA in almost all magnocellular neurons of the PVN, especially in the medial magnocellular division, and in the dorsal portion of the SON (Fig. 5a, b). On the other hand, AVP immunoreactive neurons were preferentially located in the lateral magnocellular division of the PVN and in the ventral portion of the SON without Bax-IR colocalization (Fig. 5c, d). Immunohistochemistry combined with NADPH-diaphorase histochemistry revealed that most Bax-like immunoreactive neurons contained NADPH-diaphorase activity in the medial magnocellular portions of the PVN (Fig. 5e). Some BAX(+)/NADPH(-)

neurons were also detected, particularly in the lateral magnocellular division of the PVN (Fig. 5f).

Colchicine-injection study

The immunoreactive density in the magnocellular neurosecretory neurons was increased by colchicine compared with that of the control animals (Fig. 6). Bax-IR in the neuronal perikarya of the PVN was significantly increased by colchicine (grayscale per area; 162.0 ± 11.3) compared with non-treated animals (129.5 ± 12.3) (Fig. 6).

EIA for serum Bax-like protein

We established a standard curve for the Bax EIA system (Fig. 7). The intra- and interassay coefficients of

Fig. 5. Colocalization study of Bax-IR and OXYmRNA using a combination of Bax immunohistochemistry and *in situ* hybridization in the PVN (a) and SON (b). Neurons that express OXYmRNA are stained with black silver grains, and Bax-like protein are stained as brown. Most magnocellular neurons show colocalization of Bax-IR and OXYmRNA. Double-labeling immunofluorescence histochemistry for Bax (red marked with Rhodamine) and AVP (green marked with FITC) in the medial magnocellular portion of PVN (c), and SON (d). Only one neuronal perikarya shows colocalization of Bax-IR with AVP (arrow). Double labeling immunohistochemistry to detect Bax-IR (stained as brown) and NADPH-diaphorase (stained as blue), revealed that most Bax-like immunoreactive neurons contain NADPH-diaphorase activity in the medial magnocellular portion of the PVN (arrows) (e). Some cells without colocalization are also present, particularly in the lateral magnocellular division of PVN (f). All scale bars = 100 μ m. V, third ventricle; oc, optic chiasm.

Fig. 7. (Left): Serum Bax-like protein concentrations measured by EIA. Bax-like protein levels in colchicine treated animals (n=8) significantly decreased as compared with non-treated controls (n=10). Values are expressed as means $\pm$ S.D. *, Significantly different from control at $P < 0.05$. (Right): Standard curve for Bax EIA system. Four-parameter regression analysis determined that $Y = (2.502 + 0.94) / (1 + (X/63.88)^{0.764})$, (Y, absorption density; X, standard Bax peptide. Correlative coefficients revealed that $r^2 = 0.996$) within measured standard Bax peptide range of 200 to 0.048 ng/ml.

variations were 12.38 and 15.21%, respectively. The additive and withdrawal test revealed ratios of 82.38 to 103.58%. Dose-responses were regressed by $Y = 0.70418 + 0.31123X$ (Y, standard Bax-peptide; X, dilution ratio), and correlation coefficients revealed $r^2 = 0.985$. Non-treated rat (n=10) serum samples measured with this EIA system contained 39.0 ± 9.6 pmol/ml of Bax-like protein. On the contrary, Bax-like protein levels in the serum of colchicine treated animals were significantly decreased (29.5 ± 5.3 pmol/ml) compared with those in non-treated animals (Fig. 7).

IV. Discussion

The present study demonstrated by several experimental procedures that Bax-like protein is produced in hypothalamic magnocellular neurons, transported through the axoplasmic flow to the posterior pituitary and released into the systemic circulation.

Sugimoto *et al.* have shown strong Bax-IR in the hypothalamic nucleus as we have, and suggested the possibility of Bax neurosecretion [23]. In the apoptotic process, induction of proapoptotic Bax protein has been considered in the apoptotic cell itself, and induces cell death by acting on mitochondria [12, 15]. Here we demonstrated that light and electron microscopic immunohistochemistry revealed Bax-like protein was present

in the magnocellular neurons of the PVN and SON and transported into nerve terminals in the posterior pituitary. In the rER of magnocellular neuronal perikaryon, Bax-like protein may be synthesized and incorporated into granulated vesicles. In the nerve terminals of the neuronal lobe, Bax-like protein was observed in the granulated vesicles that adjoined the pericapillary space. More precise evidence with respect to the transportation of Bax-like protein through axoplasmic flow from the neuronal perikaryon to the nerve terminal was obtained using colchicine treatment. That induced an increase in Bax-IR in the cell body of the MaPVN. Another important finding was that neurosecretion into the systemic circulation was confirmed by measuring Bax-like protein in the serum using EIA. The amount of Bax-like protein in the serum was decreased by colchicine treatment compared with that in non-treated control animals. Of course, the possibility remains that there might be other productive sources of Bax-like protein because the Bax-like protein still existed in the serum after treatment with colchicine. The colchicine treatment might also affect nervous systems in brain regions other than the hypothalamo-posterior pituitary system, and indirectly modify the function of the other Bax-like protein productive source. Anyway finally it is strongly suggested that the hypothalamo-posterior pituitary system is involved in the Bax-like protein production by the electron microscopic observations.

It is necessary to determine whether Bax protein itself or its sub-molecular components are secreted into the vessels. There is still a possibility that we have observed Bax-like components. However we propose that histochemically revealed Bax might be Bax protein itself because of certain evidence which includes the results of the immunohistochemical absorption test, the absence of a Bax homologue in the database, the agreement of the immunoblot data with the immunohistochemical findings, the presence of only a single band in the Western blots and the fact that this band corresponded to the molecular weight of Bax- α protein (21 kDa).

Double labeling histochemistry showed that Bax-like immunoreactive magnocellular neurons were Bax(+)/OXY(+), and many of Bax(+)/NADPH(+) neurons were also observed. Therefore, we consider that Bax-like immunoreactive magnocellular neurons are oxytocinergic and most of them are simultaneously nitro-oxidergic. Another characteristic finding of Bax-like immunoreactive magnocellular neurons is that these neurons are not vasopressinergic in normal conditions. The above findings mean that Bax-like protein in oxytocinergic and/or nitro-oxidergic neurons may participate in the function of OXY and/or nitric oxide in target organs or in the intracellular functions of those neurons, but may have little effect on the osmoregulated neurohypophysial secretion. Further investigations will reveal the nature of the interaction between Bax and OXY/NOS or other neuropeptides in the same neurons.

Bax knockout mice have proved viable, and Bax deficiency using knockout mice has led to a reduction in the extent of naturally occurring developmental neuronal death [5]. Bax deficient males were infertile because of germ cell death, and displayed lymphoid hyperplasia, but have not examined neuroendocrine dysfunction [9].

So far, immunohistochemical Bax immunoreactive neurons have been identified in several portions of the central nervous system, including the Purkinje cells of the cerebellar cortex and large neurons of the cerebral cortex [6, 10]. However, the adult mammalian central nervous system does not undergo active cell turnover [13, 19, 22]. This fact supports that hypothesis Bax in the brain of adult mammals is probably not involved in neuronal apoptosis under physiologically normal conditions. In the present study, Bax-IR is most prevalent in the nervous system of hypothalamo-posterior pituitary under non-pathologic conditions, but no characteristics of neuronal cell death such as mitochondrial damage occurred in the neurons of the magnocellular portions of PVN and SON. Therefore, Bax-like protein in the magnocellular neurons of the hypothalamo-posterior pituitary system is considered to not be involved in neuronal cell death with counteracting Bcl-2 protection against apoptotic cell death. Some Bax-like immunoreactive neurons were observed in the paraventricular dorsomedial cap and posterior sub-nucleus. Since neurons in these regions are known to send axons mainly to the brainstem and spinal cord [1], Bax-

like protein in these neurons of the above portion of the PVN may have other functions than neuroendocrine. We also showed Bax-like protein in the systemic circulation. Further investigation is necessary to detect Bax-like protein receptors in cells of the peripheral tissues with respect to target organs if they act as a neurohormone.

We demonstrated that Bax-like protein is normally and constantly produced in the hypothalamic PVN and SON, and secreted in the systemic circulation through the axoplasmic flow, therefore it may be related to regulating and maintaining physical homeostatic state in the peripheral tissues.

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