Neurochemistry International 57 (2010) 884–892

Contents lists available at ScienceDirect

Neurochemistry International

journal homepage: www.elsevier.com/locate/neuint

Changes and role of adrenoceptors in PC12 cells after phenylephrine administration and apoptosis induction

Lubomira Lencesova [a,b], Marta Sirova [a], Lucia Csaderova [b], Marcela Laukova [c], Zdena Sulova [a], Richard Kvetnansky [c], Olga Krizanova [a,*]

[a] Institute of Molecular Physiology and Genetics, Center of Excellence for Cardiovascular Research, Slovak Academy of Sciences, Vlarska 5, 833 34 Bratislava, Slovak Republic
[b] Molecular Medicine Center, Slovak Academy of Sciences, Bratislava, Slovak Republic
[c] Institute of Experimental Endocrinology, Slovak Academy of Sciences, Bratislava, Slovak Republic

ARTICLE INFO

Article history:
Received 21 July 2010
Received in revised form 21 September 2010
Accepted 24 September 2010
Available online 1 October 2010

Keywords:
Adrenergic receptors
Apoptosis
Noradrenaline transporter
Phenylephrine
PC12 cells

ABSTRACT

The present study addresses the hypothesis that adrenergic regulation modulates the effect of apoptosis. Therefore we studied, whether α1-adrenergic receptor's agonist phenylephrine (PE) can affect or induce apoptosis in rat pheochromocytoma (PC12) cells. We have shown that PE treatment did not increase level of the apoptosis, or level of the caspase 3 mRNA. When apoptosis was induced in the presence of PE, caspase 3 mRNA was significantly increased, while the percentage of apoptotic cells remained unchanged compared to apoptotic group without PE. During this process, α1D-, β2- and β3-adrenergic receptors (ARs) were upregulated. Since all these three types of ARs are differently localized in the cell, we assume that mutual communication of all three ARs is crucial to participate in this signaling and during development of apoptosis, some of these systems might translocate. Another important system in handling noradrenaline during apoptosis might be noradrenaline transporter (NET), since it was downregulated in apoptotic cells treated with PE, compared to untreated apoptotic cells. However, precise mechanism of mutual communication among all these systems remains to be elucidated.

1. Introduction

Noradrenaline (NA) is an important adrenergic neurotransmitter. At large doses, NA has been shown to induce cell apoptosis in a variety of cell types, including neurons, cardiomyocytes and pheochromocytoma cells (Mao et al., 2006). Recent studies have shown that the proapoptotic effect of NA involves activation of caspases 3 and 9, but the exact cellular targets and related gene expressions induced by NA are still unknown (Fu et al., 2004). Both, NA and adrenaline (Adr) bind to adrenergic receptors (ARs). ARs are members of the G-protein coupled receptor family and they mediate physiological responses to the catecholamines noradrenaline and adrenaline. ARs are subdivided into three major families (α1, α2 and β) based on their structure, pharmacology and signaling mechanisms (Hieble et al., 1995). Lands et al. (1967) subdivided the β-AR mediated effects into β1 and β2 on the basis of the rank order of potency of NA and Adr in different tissues. Later, third type of β-ARs, β3-AR, was founded and characterized (for review see Skeberdis, 2004). ARs vary in the sensitivity to Adr/NA and also in the activation of downstream cascades (for review see Krizanova et al., 2007).

Apoptosis, or programmed cell death, plays an indispensible role in embryonic development, maturation of the immune system, and maintenance of tissue and organ homeostasis (Martin, 1993). Involvement of catecholaminergic modulation in the process of apoptosis is still under investigation. Most of apoptotic effects of NA and Adr were shown on cardiomyocytes. In heart failure, apoptosis of cardiac myocytes in response to NA is believed to be an important component of the progression of cardiac fibrosis (Iwase et al., 1996; Communal et al., 1998). Stimulation of β1-AR in the rat heart can cause not only positive inotropic effects, but also can evoke cardiomyocyte apoptosis, while stimulation of β2-ARs appears to be antiapoptotic (Communal et al., 1999). Also, NA induces dose-dependent apoptosis in human and rat alveolar epithelial cells by a mechanism that involves the combination of α- and β-ARs as well as autocrine angiotensin II production (Dincer et al., 2001). It was already shown that NA activated a mitochondrial apoptotic pathway, as evidenced by the increased cleaved 37 kDa caspase 9, as well as cytochrome c translocation from the mitochondria to the cytosol in PC12 cells (Mao et al., 2006). However, it is not clear, which ARs are involved in the activation of apoptotic pathways by NA in PC12 cells.

Noradrenaline transporter (NET) is a member of gene family of Na^+/Cl^--dependent plasma membrane transporters, which removes NA from the synaptic space and quickly terminates the

* Corresponding author. Tel.: +421 2 54772211; fax: +421 2 54773666.
E-mail address: olga.krizanova@savba.sk (O. Krizanova).

0197-0186/$ – see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.neuint.2010.09.007

actions of extracellular NA on postsynaptic receptors (Blakely et al., 1994). It was already shown that decrease of NET density and NA uptake activity occurs also in PC12 cells after NA administration and these effects of NA are mediated via a posttranscription event caused by NA-derived oxidative stress (Blakely et al., 1994). Thus, NET plays a major role in the regulation of NA related actions at cellular level. Due to its early decrease in neurodegenerative diseases, NET appears to be a useful biomarker of these diseases. There are significant decreases in NET density in the LC and in Alzheimer's disease brains as compared to age matched controls (Gulyas et al., 2010).

The present study addresses the hypothesis that adrenergic modulation potentiated the effect of apoptosis in PC12 cell line. PC12 cells are derived from rat pheochromocytoma (Greene and Tischler, 1976). Pheochromocytomas are tumors of chromaffin cells that produce and often secrete catecholamines. Pharmacological control of the physiological and pathological effects of excess circulating catecholamines represents a continuing requirement in the treatment of metastatic or incompletely respectable invasive pheochromocytomas. Alpha adrenergic blockers and calcium channels antagonists often reduce hormone-mediated symptoms sufficiently. We studied, whether phenylephrine (PE), an α1-AR's agonist can induce and/or affect apoptosis in rat PC12 cells and whether PE and induction of early stage of apoptosis (for 3 h) will affect gene expression and protein levels of ARs. Also, we used immunofluorescence staining and confocal laser scanning to determine the cellular and subcellular locations of selected ARs to propose the role of these receptors in the process of apoptosis.

2. Materials and methods

2.1. Cell culture

PC12 cells were cultured in Minimal essential medium of Dulbecco (DMEM; Biochrom AG, Germany) with high glucose (4.5 g/l), supplemented with 15% fetal bovine serum (Biochrom AG, Germany) and antibiotics penicillin and streptomycin (0.5%; Calbiochem, Merck Biosciences, Germany). PC12 cells were cultured in a water-saturated atmosphere at 37 °C and 5% CO_2.

2.2. Treatment of cells with PE and AIK

PC12 cells were pretreated with 10 μM phenylephrine hydrochloride (PE; Sigma–Aldrich, Germany) for 3 h. Apoptosis inducer set I (AIK; Calbiochem, Merck Biosciences, Germany) was added to induce apoptosis in PC12 cells in the dilution 1:1000 as recommended by the provider. AIK is composed of following inducers – actinomycin D, camptothecin, cycloheximide, dexamethasone and etoposide. Apoptosis was induced for 3 h, afterwards cells were used for RNA isolation, Western blot analysis and immunofluorescence.

2.3. Measurement of pHi by fluorescence probe

Intracellular pH (pHi) was measured using the fluorescent probe 2′,7′-biscarboxyethyl-5,6-carboxyfluorescein (BCECF; Sigma–Aldrich, USA). Cells plated onto 6-well plates were loaded with 8.2 μM BCECF and 5% pluronic acid in PBS buffer, pH 7.48 for 30 min at 37 °C, 5% CO_2, in dark. Afterwards, cells were washed with PBS buffer and calibration was performed using PBS/HEPES buffers with different pH values (pH 7.51; pH 7.48; pH 7.03; pH 6.52; pH 6.01). The fluorescence was excited at 489 nm and measured at 525 nm on the fluorescence scanner BioTek (Germany). The pHi signal was calibrated to pH0 by adding 10 μM nigericin (Sigma–Aldrich, USA) with 130 mM KCl. These values were used for the calibration curve, from which different pHi values were calculated.

2.4. RNA preparation and relative quantification of mRNA levels by RT-PCR

Population of total RNAs was isolated by TRI Reagent (MRC Ltd., OH, USA). Briefly, cells were scraped and homogenized by pipette tip in sterille water and afterwards TRI Reagent was added. After 5 min the homogenate was extracted by chloroform. RNAs in the aqueous phase were precipitated by isopropanol. RNA pellet was washed with 75% ethanol and stored in 96% ethanol at −70 °C. The purity and integrity of isolated RNAs was checked on GeneQuant Pro spectrophotometer (Amersham Biosciences, United Kingdom). Reverse transcription was performed using 1.5 μg of total RNAs and Ready-To-Go You-Prime First-Strand Beads (GE Healthcare-Life Sciences, UK) with pd(N6) primer. PCR specific for the type α1-AR was carried out afterwards using primers ALPHA1-a: 5′-CGA GTC TAC GTA GTA GCC-3′ and ALPHA1-b: 5′-GTC TTG GCA GCT TTC TTC-3′, for the type α2-AR using primers ALPHA2-a: 5′-GCG CCT CAG AAC CTC TTC CTG GTG-3′ and ALPHA2-b: 5′-GAG TGG CGG GAA AAG GAT GAC GGC-3′. PCR specific for the type β1-AR was carried out afterwards using primers BETA1-a: 5′-GCC GAT CTG GTC ATG GGA-3′ and BETA1-b: 5′-GTT GTA GCA GCG GCG CG-3′, for the β2-AR using primers BETA2-a: 5′-ACC TCC TTC TTG CCT ATC CA-3′ and BETA2-b: 5′-TAG GTT TTC GAA GAA GAC CG-3′and for β3-adrenergic receptor using primers BETA3-a: 5′-GCA ACC TGC TGG TAA TCA CA-3′, BETA3-b: 5′-GGA TTG GAG TGA CAC TCT TG-3′. Cyclophilin A (CYCLO) was used as a housekeeper gene control for semi-quantitative evaluation of PCR. Following primers for the cyclophilin A were used: CYCLO FW: 5′-CGT GCT CTG AGC ACT GGG GAG AAA-3′ and CYCLO RE: 5′-CAT GCC TTC TTT CAC CTT CCC AAA GAC-3′ (Gene ID: 203701). PCR specific for α1, α2A, β1, β2 and β3 started by initial denaturation at 94 °C and was followed by 38 cycles of denaturation at 94 °C for 1 min, annealing at 60 °C for 1 min and polymerization at 72 °C for 1 min. PCR specific for CYCLO started by initial denaturation at 94 °C and was followed by 22 cycles of denaturation at 94 °C for 1 min, annealing at 60 °C for 1 min and polymerization at 72 °C for 1 min. PCRs were terminated by final polymerization at 72 °C for 7 min. All PCR products were analyzed on 2% agarose gels. Intensity of individual bands was evaluated by measuring the optical density per mm^2 and compared relatively to the CYCLO on PCBAS 2.0 software.

2.5. Western blot analysis

The α1A-, α1D-, β1-, β2- and β3-AR proteins were determined in the crude membrane fraction from the cells. Cells were scraped and resuspended in 10 mM Tris–HCl, pH 7.5, 1 mM phenylmethyl sulfonylfluoride (PMSF, Serva, Germany), protease inhibitor cocktail tablets (Complete EDTA-free, Roche Diagnostics, Germany) and subjected to centrifugation for 10 min at 10,000 × *g* and 4 °C. The pellet was resuspended in Tris-buffer containing the 50 μM CHAPS (3-[(3-cholamidopropyl)dimethyl-ammonio] 1-propanesulfonate, Sigma, USA), and afterwards incubated for 10 min at 4 °C. The lysate was centrifuged for 10 min at 10,000 × *g* at 4 °C. Protein concentration of supernatants was determined by the method of Lowry et al. (1951). 20 μg of protein extract from each sample was separated by electrophoresis on 10% SDS polyacrylamide gels and proteins were transferred to Hybond-P membrane using semidry blotting (Owl, Inc., USA). Membranes were blocked in 5% non-fat dry milk in Tris-buffered saline with Tween 20 (TBS-T) overnight at 4 °C and then incubated for 1 h with appropriate primary antibody. Following washing, membranes were incubated with secondary antibodies to mouse, rabbit or goat IgG conjugated to horseradish peroxidase, for 1 h at room temperature. An enhanced chemiluminiscence detection system (ECL Plus, Amersham Biosciences) was used to detect bound antibody. Optical density of individual bands was quantified using PCBAS 2.0 software.

Antibodies: Antibodies raised against the following proteins were used: α1D-AR (goat, Santa Cruz Biotechnology, Inc., USA), α1A-AR (goat, Santa Cruz Biotechnology, Inc., USA), β1-AR (rabbit, Santa Cruz Biotechnology, Inc., USA), β2-AR (rabbit, Abnova, USA), β3-AR (rabbit, Alpha Diagnostic International, USA).

2.6. Immunofluorescence

PC12 cells were plated on poly-L-lysine (10 mg/ml; Sigma–Aldrich, St. Louis, MD, USA) coated coverslips (Marienfeld GmbH & Co.KG, Germany) in 24-well plates in 0.5 ml of DMEM with 15% of fetal bovine serum and mixture of streptomycin and penicillin (Calbiochem, Merck Biosciences, Germany). Cells were incubated in a humidified atmosphere of 5% CO_2 air at 37 °C. After the treatment procedures, cells were fixed in ice-cold methanol. Non-specific binding was blocked by incubation with phosphate-buffered saline (PBS) containing 3% BSA (Merck Biosciences, Germany) for 1 h at 37 °C. Afterwards, cells were incubated with corresponding primary antibodies diluted 1:250 for 60 min at 37 °C. We used primary rabbit polyclonal antibodies to β2-AR (Abnova, USA), β3-AR (Alpha Diagnostic International, USA), α1D-AR (Santa Cruz Biotechnology, Inc., USA) and NET (Chemicon International, USA), coverslips were washed in PBS and incubated with CFTM488 goat anti-rabbit IgG (H + L) (Biotium, Hayward, USA) and Alexa fluor 594 rabbit anti-goat IgG (Invitrogen, USA) secondary antibody for 60 min at 37 °C. Finally, cells were mounted onto slides in mounting medium with Citifluor (Agar Scientific Ltd., UK), analyzed by fluorescent microscope Leica DM450B with Leica DFC 480 and software Leica IM 500 also by inverted confocal microscope Zeiss Axiovert 200 M with LSM510 expert mode program. Micrographs were taken at 63× magnification with optical zoom 2. Micrographs were deconvolved in Huygens Essential software (SVI, Netherlands) and analyzed in ImageJ software Volume Viewer.

2.7. Detection of apoptosis with Annexin-V-FITC

PC12 cells were washed with PBS and pelleted 200 × *g* for 5 min. Cell pellet from each well was resuspended in 100 μl of Annexin-V-FITC labeling solution and incubated at room temperature in dark for 20 min. Labeling solution contained incubation buffer with 10 mM HEPES/NaOH pH 7.4, 140 mM NaCl and 5 mM $CaCl_2$, 2 μl of Annexin-V-FITC (Roche Diagnostics, Germany) and 0.02 μg propidium iodide. After the incubation, cells were washed with 5 ml of PBS, pelleted at 200 × *g*