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Lecture

Intranuclear 3'-phosphoinositide metabolism and apoptosis protection in PC12 cells

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Abstract. Lipid second messengers, particularly those derived from the polyphosphoinositide metabolism, play a pivotal role in multiple cell signaling networks. Phosphoinositide 3-kinase (PI3K) generates specific 3'-phosphorylated inositol lipids that have been implicated in a multitude of cell functions. One of the best characterized targets of PI3K lipid products is the serine/threonine protein kinase Akt (protein kinase B). Recent findings have implicated the PI3K/Akt pathway in cancer progression because it stimulates cell proliferation and suppresses apoptosis. Evidence accumulated over the past 15 years has highlighted the presence of an autonomous nuclear inositol lipid cycle, and strongly suggests that lipid molecules are important components of signaling networks operating within the nucleus. PI3K, its lipid products, and Akt have also been identified at the nuclear level. In this review, we shall summarize the most updated findings about these molecules in relationship with suppression of apoptotic stimuli in PC12 cells. (www.actabiomedica.it)

Key words: PtdIns (3,4,5)P_3, PI3K, Akt, nucleus, apoptosis

Introduction

The transfer of signals from the plasma membrane to the cell nucleus is an exceedingly complex multistep process which strongly depends, among other components, on phosphatidylinositol (PtdIns) lipid signaling molecules (1). The bulk of inositol lipids reside in cell membranes where they are substrates for kinases, phosphatases, and phospholipases (2). The lipid kinase PI3K has emerged as an important constituent of multiple signaling pathways and as such it is involved in the control of many critical cell responses (3, 4). It synthesizes four species of non-canonical, 3'-phosphorylated inositides: PtdIns(3)P, PtdIns(3,4)P_2, PtdIns(3,5)P_2, and PtdIns(3,4,5)P_3. Compelling evidence suggests that members of PI3K family can also be considered as oncogenes, because they control cell cycle progression, differentiation, survival, invasion and metastasis, and angiogenesis (5). Several biological effects of PI3K are mediated through the activation of the downstream target Akt, a serine/threonine protein kinase, which belongs to the family of the AGC protein kinases (6).

Most of the research on inositide-dependent signal transduction pathways has focused on events that take place at the plasma membrane. However, phosphoinositides and their biosynthetic machinery are localized in the nucleus (7-9). The regulation of the nuclear inositol lipid pool is largely independent from that of the plasma membrane, suggesting that the nucleus constitutes a functionally distinct compartment for phosphoinositide metabolism (7-9). Also 3'-phosphorylated inositides, PI3K, and Akt have been reported to be present in the nucleus (10). In this article, we shall highlight the existing knowledge about the role played by these molecules in the nucleus in relationship with anti-apoptotic signaling elicited by nervous growth factor (NGF) in PC12 cells. Howe-

ver, it is firstly necessary to briefly review some general data about 3'-phosphorylated inositides, PI3K, and Akt.

3'-phosphorylated inositol lipids and PI3K

Resting mammalian cells contain significant levels of PtdIns(3)P, but hardly any of the other 3'- phosphorylated inositides. While the overall levels of PtdIns(3)P do almost not increase upon cell stimulation, the levels of the other 3'-phosphorylated inositides can rise sharply (11). Even though these lipids are not the target of any known phospholipases, they are metabolized by phosphatases that act on the inositol ring. PTEN (phosphatase and tensin homologue deleted on chromosome 10) is a 3'-phosphatase which has received a lot of attention recently, because of its role as a tumor suppressor gene (12). PTEN converts PtdIns(3,4)P_2 to PtdIns(4)P, and PtdIns(3,4,5)P_3 to PtdIns(4,5)P_2. In a significant number of human cancers, PTEN is mutated and/or inactivated so that the PI3K signaling pathway is constitutively activated as a result of the high PtdIns(3,4,5)P_3 levels (13). Two other phosphatases, SHIP-1 and SHIP-2 (for Src Homology domain-containing Inositol Phosphatases), are capable of removing the 5-phosphate from (3,4,5)P_3 to yield PtdIns (3,4,)P_2 (14).

There are multiple isoforms of PI3K in mammalian cells, and these are subdivided into three classes, referred to as I, II, and III (15, 16). Our review will focus on class I_A PI3Ks which are the most studied because they are generally coupled to extracellular stimuli. They display a preference in vivo for PtdIns(4,5)P_2. Class I_A PI3Ks are heterodimeric enzymes composed of a p110 catalytic subunit (α, β, and δ) and an adaptor/regulatory subunit. There are at least seven adaptor proteins that are generated by expression and alternative splicing of three different genes (p85α, p85β, and p55γ).

Akt

At present, three members of the Akt family have been identified and are referred to as Akt1, Akt2, and Akt3. Although they are products of different genes, they are highly related exhibiting more than 80% sequence homology (6, 17). In response to a variety of stimuli (hormones, growth factors, cytokines), inactive (cytosolic) Akt is recruited to the plasma membrane by the products of PI3K, PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3. Then, Akt is phosphorylated at threonine 308 (by a phophoinositide-dependent kinase 1, or PDK1 (whose activity strictly depends on 3'-phosphorylated inositol lipids, see Ref. 18) and at serine 473 by a still undefined kinase. This double phosphorylation fully activates Akt (17). A plethora of Akt substrates have been identified and these include, among the others, BAD, CREB, members of the forkhead family of transcriptions factors, Iκ-B kinase, procaspase-9, GSK-3-α/β, mTOR/FRAP, $p21^{WAF1}$ (17). The large variety of proteins that are phosphorylated by Akt explains why this kinase has rapidly emerging as a key mediator of cell proliferation, differentiation and survival. Moreover, increasing evidence points to the likelihood that Akt plays an important role in tumorigenesis and resistance to chemotherapeutic drugs (19, 20).

Nuclear 3'-phosphorylated inositol lipids and class I_A PI3Ks

The presence of these inositol lipids in the nuclear compartment has been demonstrated by means of different techniques (radioisotope labeling, immunocytochemistry) in a variety of cell types, including PC12 cells (10). Consistently with the presence of 3'-phosphorylated inositol lipids, the nucleus possesses proteins that bind these lipids, such as a 43-kDa PtdIns(3,4,5)P_3-binding protein containing one zinc finger motif and two pleckstrin homology domains (21). The first report dealing with PI3K at the nuclear level, demonstrated a rapid translocation of PI3K to the nucleus of PC12 rat pheochromocytoma cells in response to NGF stimulation (22). Subsequently, intranuclear class I_A PI3K has been detected in other cell types, including Saos-2 human osteosarcoma cells, rat hepatocytes, Hep-G2 human hepatocarcinoma cells, and HL60 human promyelocytic leukemia cells (10). HL60 cells are a unique experimental model because

they differentiate into granulocytic cells in response to retinoids. In contrast, treatment with vitamin D3 induces HL60 cell maturation into monocytes (23). Nuclear class I_A PI3K might play a relevant role in the differentiation process of HL60 cells. Indeed, retinoids (24) or vitamin D3 (25) induced a striking increase in nuclear matrix-bound p85α regulatory subunit of PI3K, an intranuclear synthesis of PtdIns(3,4,5)P_3, and an up-regulation of nuclear PI3K activity which paralleled granulocytic and monocytic differentiation. Interestingly, no significant variations were detectable in cytoplasmic PI3K activity. Moreover, HL60 cell differentiation was prevented by either PI3K activity inhibition by wortmannin or the expression of p85α antisense oligonucleotides (24).

The regulation of nuclear class IA PI3K activity in PC12 cells

While control of cytoplasmic class I_A PI3K is quite well defined (e.g. 15, 16), regulation of its nuclear counterpart has been obscure. A major breakthrough has been achieved in PC12 cells stimulated with NGF. By means of a yeast two-hybrid approach, Ye et al. (26) identified the protein PIKE (PhosphoInositide 3-Kinase Enhancer) as a novel physiological regulator of nuclear class I_A PI3K. PIKE is a nuclear GTPase characterized by a PX domain and three proline-rich domains, which typically bind to SH3 domains of target proteins. Retroviral infection of PC12 cells showed that NGF-induced nuclear PI3K activity was blocked by a dominant-negative form of PIKE, and that PI3K activation by PIKE was GTP-dependent and required the presence of both p85 and p110 subunit. Subsequently, the same group identified nuclear phosphoinositide-specific phospholipase C (PI-PLC) γ1 as the guanine nucleotide exchange factor (GEF) for PIKE (27). Indeed, the SH3 domain of PI-PLCγ1 directly bound the third proline-rich domain (amino acids 353-362) of PIKE and this interaction stimulated GDP dissociation, markedly enhanced GTP binding to PIKE, and was required for nuclear PI3K activation. This finding might partly explain the puzzling observation that the mitogenic activity of PI-PLCγ1 does not actually require it to be catalytically active, but does indeed require the SH3 domain to be present (28). In addition, the same authors have suggested that down-regulation of nuclear PI3K activity could result from the interaction between PIKE and the protein 4.1N (26). Indeed, in NGF-treated PC12 cells, they observed protein 4.1N translocation to the nucleus with a slower time course than for PI3K translocation and PIKE activation. The binding of the protein 4.1N to PIKE inhibited PIKE GTPase activity and prevented association between PIKE and PI3K, resulting in nuclear PI3K activity decrease (Figure 1).

Nuclear Akt

It is now clear that phosphorylated (active) Akt migrates to the nucleus (see Figure 1). Indeed, some of its substrates are resident within this organelle, such as the FoxO family of transcription factors (29). Either Akt1 or Akt2 have been reported to migrate into the nucleus in response to a variety of stimuli including serum, activation of B-lymphocytes, hypoglycemic coma, mitogenic stimulation with polypeptide growth factors, differentiating treatment with NGF (reviewed in 10). It should be underscored that, apart from FoxO factors, no other nuclear substrates of Akt have been identified yet. We have no doubt, however, that other nuclear Akt substrates will emerge because the minimal consensus peptide phosphorylation sequence of Akt has been identified in more than 400 different proteins, some of which are resident in the nucleus, such as lamin A/C (30).

Involvement of 3-phosphorylated inositol lipid metabolism in NGF-DEPENDENT anti-apoptotic signaling of PC12 cells

PI3K/Akt pathway is by far the most important signaling network for cell survival (31). Traditionally, anti-apoptotic signaling by PI3K/Akt has been thought to take place at the plasma membrane level and in the cytoplasm (32). However, recent findings point to the likelihood that also nuclear PI3K plays an essential role in promoting cell survival through nuclear PtdIns (3,4,5)P_3.

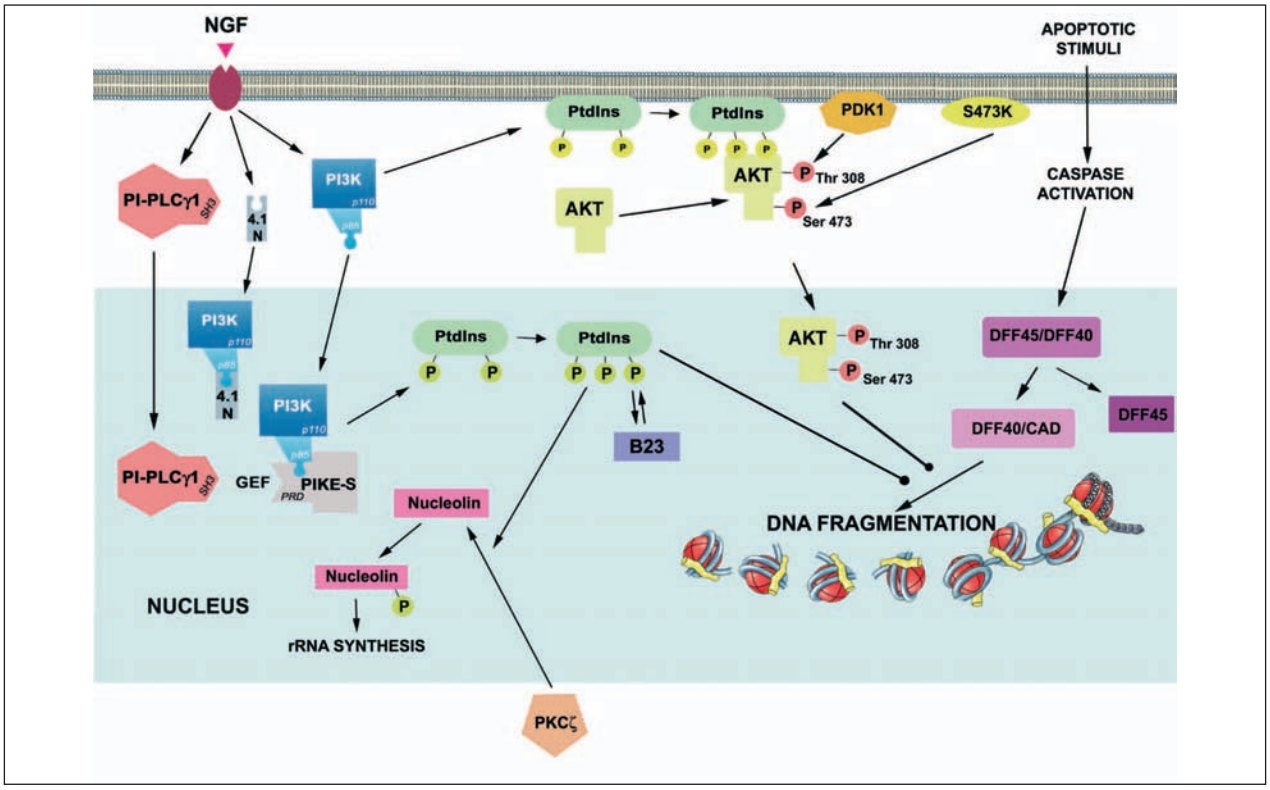

Figure 1. NGF elicits nuclear PtdIns(3,4,5)P_3-dependent anti-apoptotic signaling in PC12 cells. In response to NGF treatment, there is intranuclear migration of a class I_A PI3K (composed of a p110 catalytic subunit and a p85α regulatory subunit). Concomitantly, also PI-PLCγ1 translocates to the nucleus. The SH3 domain of PI-PLCγ1, acting as GEF, stimulate the activity of the nucleus-specific GTPase, PIKE. The target of the PI-PLCγ1 SH3 domain is one of the three proline-rich domains (PRD) of PIKE. PIKE then interacts with the p85α subunit of PI3K, whose activity is stimulated. PI3K synthesizes intranuclear PtdIns(3,4,5)P_3 from PtdIns(4,5)P_2. Later on, protein 4.1N enters the nucleus to down-regulate PI3K activity. In non-apoptotic cells, DFF exists in the nucleus as a heterodimer, composed of a 45-kDa chaperone and inhibitor subunit (DFF45) (also called inhibitor of CAD (ICAD-L)) and a 40-kDa latent nuclease subunit (DFF40/CAD). Apoptotic activation of caspase-3 or -7 results in the cleavage of DFF45/ICAD and release of active DFF40/CAD nuclease (see ref. 51). PtdIns(3,4,5)P_3, through its interaction with B23/nucleophosmin, blocks DFF40/CAD-dependent DNA fragmentation. To this end, phosphorylated (activated) nuclear Akt is also necessary but not sufficient. Akt recruiting at the plasma membrane requires PtdIns(3,4,5)P_3 synthesized by receptor-associated PI3K, while its phosphorylation is dependent on PDK1 and another, as yet unidentified, protein kinase (S473K). PtdIns(3,4,5)P_3 attracts to the nucleus PKC-ζ which resides in the cytoplasm of unstimulated cells. Once in the nucleus, PKC-ζ phosphorylates the multifunctional nucleolar protein nucleolin. Nucleolin phosphorylation is known to up-regulate rRNA synthesis.

The PC12 cell line was originally derived form rat pheochromocytoma and can be differentiated by means of NGF (33). Differentiated PC12 cells display proliferation arrest and neurite outgrowth. In PC12 cells, NGF treatment also elicits powerful anti-apoptotic signaling cascades through binding to the tyrosine kinase receptor, TrkA (34, 35). PI3K migrates to the PC12 cell nucleus in response to NGF (36). Taking advantage of a cell-free system, it has been shown that nuclei isolated from NGF-treated PC12 cells were resistant to DNA fragmentation factor/caspase activated DNase (DFF40/CAD) -dependent DNA cleavage initiated in vitro by activated cell-free apoptotic solution, consisting of HEK293 cell cytosol supplemented with purified active caspase-3 (37). Nuclei from constitutively active PI3K adenovirus-infected cells displayed the same resistance as those treated with NGF, whereas PI3K pharmacological inhibitors, immunodepletion of PI3K from nuclear extracts with anti-p110 antibody, and dominant negative PI3K or

PIKE abolished it. PtdIns (3,4,5)P_3 alone, but not PtdIns (3,4)P_2, PtdIns (4,5)P_2 or PtdIns (3)P, mimicked the anti-apoptotic effect of NGF. The involvement of nuclear PtdIns (3,4,5)P_3 in the protecting role of NGF was also substantiated by an experiment in which isolated nuclei were preincubated with PTEN (which dephosphorylates PtdIns (3,4,5)P_3 to PtdIns (4,5)P_2) and then analyzed for DNA fragmentation. It was found that PTEN pre-treatment abolished the protective effect of NGF, even though it was not demonstrated that PTEN actually decreased the amount of PtdIns (3,4,5)P_3 (37). In this connection, a good control would have been constituted, in our opinion, of a mutated PTEN lacking the lipid phosphatase activity (38). Since NGF treatment stimulates migration of phosphorylated Akt to the nucleus of PC12 cells (39), the role of nuclear Akt in the anti-apoptotic action of NGF was also examined. It turned out that nuclei isolated from cells overexpressing wild type or constitutively active Akt were resistant to internucleosomal DNA cleavage, whereas those from dominant-negative Akt-infected cells showed DNA cleavage in spite of NGF treatment, demonstrating that nuclear Akt is required for NGF-mediated anti-apoptotic signaling (Figure 1). Nevertheless, in the absence of NGF treatment, all the nuclei displayed DNA degradation, suggesting that Akt activation alone is not sufficient to inhibit DNA cleavage (37). The down-stream effectors of nuclear PtdIns (3,4,5)P_3 which prevent apoptosis has been recently identified as B23/nucleophosmin, a major nucleolar protein (40).

Furthermore, it is worth recalling here that PIKE mediates NGF-dependent cell survival also in intact cells, because apoptosis induced by staurosporine was much higher in PC12 cells in which PIKE has been knocked down by means of antisense oligonucleotides (37).

It has also been reported that in NGF-stimulated PC12 cells, the nuclear translocation of protein kinase C (PKC) -ζ was strictly dependent on PtdIns(3,4,5)P_3 generated in the nucleus by PI3K (36). PKC-ζ, once in the nucleus of PC12 cells, phosphorylates nucleolin (41), a multifunctional nucleolar protein involved in several aspects of rRNA metabolism and transport. In particular, phosphorylation of nucleolin is known to increase rRNA synthesis rate (42). We do not know if nucleolin phosphorylation is somehow related to the anti-apoptotic effect exerted by NGF on PC12 cells. It is intriguing, however, that nucleolin has been described as a stabilizing agent for the anti-apoptotic protein, Bcl-2 (43, 44) and that apoptosis is accompanied by a decrease in the levels of nucleolin (45). Therefore, phosphorylated nucleolin might be another key player in the nuclear events which underlie the anti-apoptotic effect of NGF in PC12 cells.

Conclusions

The significance of the existence of an autonomous inositol lipid metabolism is still unclear. Divecha et al. (46) originally hypothesized that the nuclear inositol lipid cycle might have evolved first to regulate functions as crucial as DNA replication and gene expression in response to environmental messages and then it was duplicated at the plasma membrane in order to allow cross-talking between extracellular signals and the genome in multicellular organisms. Traces of this evolution have been left, represented by the inositol lipid cycle associated with the cytoskeleton (47). This pioneering hypothesis has now started receiving support from experimental data showing that phosphoinositides are involved in maintaining chromatin in a transcriptionally active conformation (48) and could act as ligands for the NR5 orphan receptors SF-1 and LHR-1 (49). Therefore, inositol phospholipids might regulate gene expression by directly binding to NR5A nuclear receptors, a class of molecules which control crucial aspects of development, endocrine homeostasis, and metabolism (49).

As to the anti-apoptotic functions of nuclear PI3K/ PtdIns (3,4,5)P_3/Akt, several issues needs to be clarified. For example, the role played by Akt is unclear. Moreover, we need to find out whether or not this pathway is also activated by other neurotrophins which protects neural cells from apoptosis, such as insulin-like growth factor-1 (50). Nevertheless, we feel that, once we will have a more detailed picture of what is going on within the nucleus, this complex and peculiar intranuclear survival pathway might constitute an interesting target for the development of therapeu-

tic strategies for neurodegenerative disorders in which inappropriate apoptosis is thought to play a fundamental role, such as Parkinson's disease and amyotrophic lateral sclerosis.

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