Cellular Signalling 21 (2009) 161–168

Contents lists available at ScienceDirect

Cellular Signalling

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

Haloperidol disrupts Akt signalling to reveal a phosphorylation-dependent regulation of pro-apoptotic Bcl-XS function

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ARTICLE INFO

Article history:
Received 25 June 2008
Received in revised form 8 October 2008
Accepted 8 October 2008
Available online 12 October 2008

Keywords:
Bcl-X
Cytochrome c
Mitochondria
VDAC
Dementia
Schizophrenia
Sigma receptor

ABSTRACT

The antipsychotic drug haloperidol is still used to treat psychosis and "agitation", often with devastating consequences, particularly in geriatric and pre-demented patients. Cytotoxicity induced by haloperidol has been associated with induction of Bcl-XS, a pro-apoptotic member of the Bcl-2 family, as well as with modulation of the Akt pro-survival pathway. Using preneuronal PC12 and primary neuronal cultures, we show that haloperidol inactivates Akt. This induces the dephosphorylation of serine residues in Bcl-XS and promotes its association with the mitochondrial voltage-dependent anion channel (VDAC), as well as with cytochrome *c*- and caspase-3-dependent events. These events are sensitive to expression of constitutively active Akt. Mutation of Serine106 (Ser106), which is flanked by a putative Akt motif, hinders the association of the Bcl-XS protein with Akt, but promotes its association with VDAC. The dephosphorylation mimic, Bcl-XS(Ser106Ala), induces caspase-dependent PC12 and neuronal cell apoptosis. In contrast, Bcl-XS(Ser106Ala) induces a significant loss of VDAC expression, and cytochrome *c*- and caspase-independent toxicity in the non-neuronal HEK293A cells. We link haloperidol and Akt to Bcl-XS-sensitive toxicity *via* cell line-dependent mitochondrial events centering on VDAC. This clearly mitigates the chronic use of haloperidol in neuropsychiatric populations, but supports its use as a potential acute therapeutic in cancer, where apoptosis is desirable.

1. Introduction

The phosphatidylinositol 3′-kinase (PI3K)/Akt pathway promotes survival *via* phosphorylation-dependent inactivation of pro-apoptotic effectors including members of the Bcl-2 family of proteins (see [1] for review; [2]). Bcl-2-related proteins contain structural features referred to as Bcl-2 homology (BH) domains. The best characterized pro-apoptotic Bcl-2 family member, Bax, has the obligatory BH3 domain in addition to membrane-spanning BH1 and BH2 domains that contribute to channel formation in synthetic membranes and lipid vesicles. In contrast, pro-apoptotic Bcl-XS, devoid of BH1 and BH2 domains and lacking the potential to form membrane-spanning channels, must act through a different mechanism. Bcl-XS does have a BH3 domain as well as a BH4 domain (thought to allow for protein–protein interactions outside the Bcl-2 family), which is a unique combination among the Bcl-2 family members [3]. The modulation of cytochrome *c* release from the mitochondria might reflect channel formation by Bcl-2 homologue oligomers, for example, Bax [4], as well as by hybrid channels formed by associating with a pore complex centered on the voltage-dependent anion channel (VDAC) [5–7].

Bcl-2-related proteins are crucial in regulating apoptosis and often exert their pro- or anti-apoptotic effects following some means of post-translational modification; this can involve, for example, cleavage (*e.g.* of Bcl-2, Bid, or Bim) or phosphorylation. Bcl-2-Thr56 can be phosphorylated during mitosis by CDK [8] and JNK [9]. This phosphorylation of Bcl-2 might disable its anti-apoptotic function [9,10], yet multisite phosphorylation might actually stabilize Bcl-2 and promote its anti-apoptotic function [11]. JNK phosphorylates Bcl-XL within its activation loop on Ser62 [12] and possibly on threonine residues [13]. Akt-mediated phosphorylation of Bax-Ser184 sequesters it in the cytoplasm away from mitochondrial targets [2]. Multisite phosphorylation of Bad-Ser112 by ERK and Ser136 by Akt [14,15], on Ser155 (by PKA, but only if Ser136 is phosphorylated: [16]) and on Ser170 [17] promotes, in part, its association with the cytoplasmic 14-3-3 proteins and suggests a need for tight control of this highly effective pro-apoptotic protein. Akt phosphorylates Bim-Ser87, which promotes its binding with 14-3-3 proteins [18]. The phosphoregulation of pro-apoptotic Bcl-XS has as yet to be assessed, although work on its anti-apoptotic variant Bcl-XL suggests that multisite phosphoregulation is highly probable.

We have demonstrated that apoptosis induced in preneuronal PC12 and neuroblastoma N2a cultures by the antipsychotic drug haloperidol (HAL) is dependent on the sigma2 (σ2) receptor, rather

Abbreviations: HAL, haloperidol; DTG, 1,3-di-*o*-tolylguanidine; SUL, sulpiride; PARP, poly(ADP-ribose) polymerase; VDAC, voltage-dependent anion channel.

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doi:10.1016/j.cellsig.2008.10.005

than the anticipated dopamine D2 receptor, and on induction and mitochondrial accumulation of Bcl-XS [19,20]. This is a p53/Bax-independent process [20]. Furthermore, we have clearly demonstrated that HAL can inactivate Akt in PC12 cells by disruption of the PI3K/Akt cascade at the level of PDK1, the upstream activator of Akt [21]. Others have also linked PI3K/Akt function to the pharmacology of antipsychotics, including HAL, and with schizophrenia [22–24].

We hypothesized that HAL-mediated toxicity results from an Akt-sensitive, Bcl-XS-mediated event. We chose to use HAL, not only because of its continued use in the clinical setting, but also because it is a useful tool for the study of apoptosis and cytotoxicity. We tested our hypothesis in the PC12 cell line which can undergo apoptosis *via* distinct Bcl-XS- and Bax-mediated cascades [25,26], and as such is a good *in vitro* model for examining the role of signalling cascades on these two Bcl-2-related proteins. Our observations in PC12 cells were compared and contrasted with those obtained for primary cortical neurons and for the non-neuronal HEK293A cell line.

2. Experimental/materials and methods

2.1. Chemicals and antibodies

Antibodies recognizing Bcl-XS/L, caspase-9 [recognizes both mature (48 kDa) and cleaved (35 kDa) proteins] and PARP were obtained from Santa Cruz Biotechnology. The VDAC antibody was from Calbiochem Anti-Bcl-X and anti-cytochrome *c* was obtained from BD Transduction Laboratories, while anti-β-actin and anti-phosphoserine, protease inhibitor cocktail, haloperidol, sulpiride and DTG were from Sigma-Aldrich, Inc. Anti-Akt and anti-caspase-3 [recognizes the cleaved (17/19 kDa) species], and the PI3K inhibitor LY294002 were from Cell Signaling Technology. IgG-HRP conjugates were from Cedarlane Laboratories. The cell permeable caspase-3 inhibitor Z-DEVD-fmk was from R&D Systems.

2.2. Immortalized cell and primary cell cultures

The PC12 rat preneuronal cell line (CRL-1721) and the HEK293 human embryonic kidney cell line (CRL-1573) were purchased from ATCC and were maintained according to their specifications.

Fetal rat (E18) primary neuronal cultures were prepared as described [27]. Timed-pregnant Sprague–Dawley rats were obtained from Charles River (St. Constant, QC) and maintained according to guidelines set by the University of Saskatchewan Animal Care Committee and the Canadian Council for Animal Care. Treatments of primary cells were performed on day 7 of culture.

2.3. Plasmids, cDNA mutagenesis and transfection

CS2+myr-Akt expresses a membrane-directed, constitutively active, *N*-myristoylated full-length mouse Akt1 (myr-Akt: a gift from Dr. A.B. Vojtek, University of Michigan, Ann Arbor). Wildtype Bcl-XS was amplified from PC12 cDNA and was subcloned into the pCMV expression vector (Invitrogen Life Technologies) or in-frame with an N-terminal triple-FLAG (a gift from Dr. D.H. Anderson, University of Saskatchewan). Bcl-XS serine-to-alanine substitutions were generated with the QuikChange® kit (Stratagene). Cells in log phase were transfected with plasmid DNA (1–2 μg/well on a 24-well plate; seeded at 5×10^5 cells/well) using LipofectAmine Plus (Invitrogen Life Technologies). Expression of eGFP revealed a transfection efficiency of 35% and 60% in PC12 and HEK cells, respectively.

2.4. Immunodetection and subcellular fractionation

Standard SDS-PAGE Western blot conditions were used to detect expression of targeted proteins in total cell lysates (precleared; 5000 ×g, 10 min, 4 °C; 20–30 μg/lane) or in immunoprecipitates (300–500 μg: precleared lysates using non-specific mouse or rabbit IgG) isolated with selected antibodies and precipitated with protein-A/G Sepharose. Detection relied on enhanced chemiluminescence (Pierce) and depicted blots are representative of 2–3 individual experiments.

Subcellular fractionation was achieved using differential centrifugation [21]. Briefly, lysates were centrifuged (900 ×*g*, 10 min, 4 °C) and the resulting supernatant was centrifuged (18,000 ×*g*, 40 min, 4 °C) to yield a mitochondrial-enriched pellet and a supernatant that represented the cytosolic [soluble] fraction.

2.5. Cell viability assays

Hoechst 33258 staining ($n\geq3$) in primary cells was used to reveal chromatin condensation, a nuclear characteristic of apoptosis. Conversion of MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium] to formazan crystals reflects mitochondrial function and cell viability in PC12 cells, as previously validated [20]. Experimental means are based on ≥6 individual experiments, each of which is represented by 4–6 replicates per test group.

2.6. Statistical analysis

Significance (set at $P<0.05$) was assessed by ANOVA and Bonferroni's Multiple Comparison Test (*post hoc*).

3. Results

3.1. HAL-induced apoptotic primary neuronal cell death is not dependent on the dopamine D2 receptor system

HAL is a ligand for the dopamine D2 receptor as well as for σ receptors in functional and binding assays. A role for the dopamine D2 receptor in the current model is precluded by the inability of the D2 receptor antagonist sulpiride (SUL), which lacks σ receptor binding affinity [28], to impact primary neuronal cell viability as measured by MTT conversion (Fig. 1a). HAL is toxic to these cells, with an LC50 value of 57 ± 16 μM (*note*, LC50 is the [lethal] concentration required to kill 50% of the test culture) (Fig. 1a). Co-treatment with either SUL or with the highly-charged (and cell impermeable) σ ligand DTG does not impact the cytotoxicity of HAL (Fig. 1b).

In primary cortical neurons, HAL induces pro-apoptotic Bcl-XS (but not pro-apoptotic Bax) expression in a time-dependent manner (Fig. 1c). The time-dependent HAL-induced inactivation of Akt (as indicated by its dephosphorylation, particularly on Thr308: Fig. 1c) precedes the appearance of pro-apoptotic events such as the cleavage of caspase-9 to the active p35 fragment as well as the cleavage of the executioner caspase-3 to the active p17 species (Fig. 1c). Apoptosis and the involvement of caspase-3 are further corroborated by concurrent cleavage of PARP to the p85 species (a caspase-3-dependent process: [29]). Staining with Hoechst 33258 reveals an increase in the number of cells exhibiting chromatin condensation (a hallmark of the terminal stages of apoptosis; Fig. 1d) in HAL-treated cultures. The number of apoptotic nuclei in cells treated with DTG or SUL is similar to that in control cultures (Fig. 1d). HAL (but not DTG) induces the expression of Bcl-XS and the dephosphorylation of Akt (Fig. 1e), and the activation of both caspase-9 and -3, as demonstrated by the increase in their respective active cleavage products (Fig. 1f).

3.2. HAL-induced apoptosis is sensitive to Akt and dependent on caspase-3 in primary neuronal cells

Expression of a myristoylated, constitutively active Akt (myr-Akt; Fig. 2c) reverses the chromatin condensation associated with HAL (a representative example is depicted in Fig. 2a and total experimental data are quantitated in Fig. 2b). Treatment with the PI3K inhibitor LY294002 inhibits Akt phosphorylation, as expected (Fig. 2d), and is toxic