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The regulation of apoptosis by the downstream regulatory element antagonist modulator/potassium channel interacting protein 3 (DREAM/KChIP3) through interactions with hexokinase I

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ABSTRACT

The EF-hand protein, DREAM/KChIP3 (henceforth referred to as DREAM), regulates apoptosis by incompletely understood mechanisms. We demonstrate that in the presence of Ca^{2+}, DREAM interacts with hexokinase I, a protein known to bind mitochondria and regulate apoptosis. A mutant DREAM protein construct incapable of binding Ca^{2+} does not associate with hexokinase I. The amino-terminal portion of DREAM is required for binding to hexokinase I, as a DREAM construct lacking the first 94 amino terminal residues fails to bind hexokinase I. Expression of DREAM in neuroblastoma cells enhances cisplatin mediated caspase-3 activity. Simultaneous expression of hexokinase I in such cells reduces DREAM-stimulated apoptosis. DREAM overexpression in neuroblastoma cells reduces hexokinase I localization on isolated mitochondria. The interaction of DREAM with hexokinase I may be important in the regulation of neuronal apoptosis.

1. Introduction

Cell number and tissue viability are regulated by apoptosis [1–5]. Altered apoptosis is noted in several diseases including cancer and neurodegenerative diseases such as Alzheimer's, Huntington's and Parkinson's diseases [1,6,7]. Understanding mechanisms by which apoptosis is regulated is important in the prevention and treatment of various neoplastic and neurodegenerative disorders.

The neuronal EF-hand protein, downstream regulatory element antagonist modulator (DREAM/Calsenilin/KChIP3) regulates diverse metabolic processes [8–11]. DREAM represses endogenous opioid production by binding to regulatory elements in the *prodynorphin* (*pdyn*) and c-*fos* genes [8] and binds to Ca^{2+}-activated Kv4 potassium channels in the brain and heart [12]. *Dream*$^{-/-}$ mice display reduced responses to pain, and improved contextual fear conditioning [13,14]. DREAM regulates apoptosis [15,16] by altering intracellular Ca^{2+} signaling [16], changing presenilin 1 and 2 activity [9,15,17] and by binding to peroxiredoxin 3, an antioxidant enzyme [18]. DREAM binds to the carboxyl-terminal portion of presenilin to regulate γ-secretase function [9]. DREAM regulates the levels of proteolytic products of presenilin 2 [9] and reverses the presenilin-mediated enhancement of calcium signaling [19]. Indeed, *dream*$^{-/-}$ mice have approximately 50% less Aβ40 and Aβ42 in the cerebellum [20]. DREAM concentrations are increased in the cortex of brains of patients with Alzheimer's disease and in the neocortex and the hippocampus of brains of mice overexpressing a Swedish mutant β-amyloid precursor protein [15]. DREAM is also a substrate for caspase-3 [10].

To gain insights into how DREAM alters apoptosis in neuronal cells, we investigated whether DREAM interacts with other previously identified regulators of apoptosis and regulates their activity. We report that DREAM interacts with brain hexokinase I in a Ca^{2+}-dependent manner, and demonstrate the functional relevance of the interaction.

Abbreviations: Ca^{2+}, calcium ion; DREAM, downstream regulatory element antagonist modulator; KChIP3, potassium channel interacting protein; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol-bis(2-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.

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2. Materials and methods

2.1. Expression and purification of DREAM protein constructs

Full length (FL) DREAM (amino acids 1–256), sFL-DREAM (amino acids 95–256), and calcium insensitive Mut-1234-DREAM (E103A,D110A, D139A,D141A, D175A,N177A, D223A,N225A), were expressed as N-terminal glutathione S-transferase (GST) fusion products in *Escherichia coli* BL21 cells and purified as previously described [21,22].

2.2. Synthesis of DREAM expression vectors

DREAM pcDNA3.1 sense and anti-sense constructs were prepared by inserting the *Eco*RI cleaved DREAM fragments obtained from the chimeric DREAM pCR2.1 plasmid into the *Eco*RI site of pcDNA3.1(-) (Life Technologies) as described earlier [23].

2.3. Expression and purification of full length hexokinase I protein

Full length human hexokinase I with a carboxyl-terminal 10X His tag (GenBank accession NM_000188.2) was expressed in the pET28a(+) vector and *E. coli* Rosetta 2 (DE3) host (EMD-Novagen, Billerica, MA). The cDNA (Origene) was amplified using the following oligonucleotides (5′ → 3′) for PCR: 5PCI1hHK1 :5′GAGA ACATGTCCATGATCGCCGCGCAGCTCCTGGCCTATTACTTCAC3′;

3HK110xHisXh:5′GAGACTCGAGTTAGTGATGGTGATGATGGTG-ATGGTGATGATGGCTGCTTGCCTCTGTGCGTAACCGCACGCCCACGG-CCGTG3′. The PCR amplified products were cleaved by *Sci*I and *Xho*I and ligated into *Nco*I/*Xho*I cleaved, dephosphorylated pET28a(+). Transformed *E. coli* Rosetta 2 (DE3) were grown in 2xYT medium with 40 μg/mL kanamycin, and 20 μg/mL chloramphenicol, initially at 37 °C to an OD 600 nm of ~1. The temperature was reduced (16 °C), and protein expression was induced for 23 h with 0.2 mM *iso*-propylthiogalactoside. Cells were lysed and protein was purified on Ni-NTA Superflow resin (Qiagen, Valencia, CA).

2.4. Synthesis of a pCMV6-AC-hexokinase I-mRFP plasmid

The hexokinase I gene was cloned into the pCMV6-AC-mRFP vector to create a vector which expressed hexokinase I with a c-terminal monomer RFP (mRFP) fusion product. The hexokinase I cDNA (OriGene, Rockville, MD) was amplified by PCR using the following primers: 5′ → 3′: 5_3gfpHK*Asc*1: GAGAGGCGCGCCATG ATCGCCGCGCAGCTCCTGGCCTAT and 3_5gfpHK*Xho*1: TCTCCTCGA GGCTGC TTGCCTCTGTGCGTAACCGC. The product was treated with restriction enzymes *Asc*I and *Xho*I (New England Biolabs, Ipswich, MA) and ligated into the pCMV6-AC-mRFP vector treated with similar restriction enzymes.

2.5. DREAM affinity capture assay

This was performed as described earlier [22]. Homogenates of rat brain (10% weight/volume) were prepared in 10 mL buffer containing 50 mM Tris–HCl (pH 7.5), 50 mM NaCl, 2 mM dithiothreitol (DTT), mini cOmplete, EDTA-free, protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN) and either 2 mM EDTA (buffer A), or 5 mM $CaCl_2$ (buffer B), respectively. Homogenates in Ca^{2+} or EDTA buffer were applied to columns prepared by incubating GST-DREAM or GST with glutathione Sepharose resin (Miltenyi Biotec, Auburn, CA). The resins were extensively washed with Ca^{2+} or EGTA buffer containing 2 M NaCl. Residual proteins bound to the column were eluted with Ca^{2+} or EGTA buffer containing 50 mM reduced glutathione. Fractions were run on an SDS–PAGE gels and were stained with 0.02% PhastGel Blue R. Mass spectrometric identification of proteins bound to GST-DREAM or GST was carried out as described in Ramachandran et al. [22]. Scaffold (version Scaffold_2_00_06, Proteome Software Inc., Portland, OR) was used to validate MS/MS based peptide and protein identification.

2.6. Assessment of DREAM hexokinase I interactions

A protein capture assay was conducted to test the binding of rat brain hexokinase I to full length, short full length, and a calcium insensitive mutant GST-tagged DREAM constructs in the presence of either EDTA or Ca^{2+} as described above [22]. GST bound to glutathione resin and resin alone, were used as controls. Resins (125 μL of each, equilibrated with 2.5 mL of either Buffer A (50 mM Tris–HCl pH 7.5, 50 mM NaCl, 2 mM DTT, 5 mM $CaCl_2$) or Buffer B (50 mM Tris–HCl pH 7.5, 50 mM NaCl, 2 mM DTT, 2 mM EDTA) were prepared. Rat brain homogenate was added to appropriate resins in the presence of EDTA or Ca^{2+} buffers. Resins were washed with either Buffer A or Buffer B, and bound proteins were eluted with either Buffers A or B containing 50 mM glutathione. Eluates were electrophoresed on a SDS gel, transferred to a PVDF membrane and analyzed by western analysis with a rabbit anti-HK I antibody (Cell Signaling Technology, Danvers, MA) and an appropriate secondary antibody (peroxidase labeled goat anti-rabbit antibody, DAKO).

2.7. Determination of hexokinase I activity

Hexokinase I activity [24] was measured at 22 °C in 96-well plates by assaying the change in absorbance of NADPH in the presence or absence of added buffer or DREAM (6–12:1 M ratio, DREAM:hexokinase I) with Ca^{2+} or EGTA present. To 80 μl assay buffer (90 mM tricine-KOH, pH 8.1, 5 mM glucose, 0.25 mM $NADP^+$ (Sigma–Aldrich, St. Louis, MO), 10 mM $MgCl_2$, 5 mM ATP, disodium salt, and glucose-6-phosphate dehydrogenase (0.0125 U) were added Ca^{2+} or EGTA (1 mM and 1.25 mM final concentrations), and 20 μl recombinant hexokinase I (20, 40 or 80 ng diluted in 0.1 M tricine pH 8.1). The absorbance of the reaction mixture was measured for 5 min at 340 nm with a SpectraMax M2 microplate reader (Molecular Devices).

2.8. Biolayer interferometry (BLI) measurements to assess the binding of DREAM to hexokinase I

Biotinylated DREAM was synthesized by adding a 10-fold molar excess of sulfosuccinimidobiotin (Thermo Scientific) to 40 μM DREAM in 50 mM Na^+/K^+ phosphate buffer, pH 7.5, 150 mM NaCl for 30 min at room temperature. Biotinylated DREAM was dialyzed against 50 mM Tris–HCl, pH 7.5, 150 mM NaCl, and 1 mM DTT buffer. Streptavidin biosensors (Pall Forte Bio, Menlo Park, CA), were hydrated in H_2O for 10 min, and then in either Ca^{2+} or EGTA containing buffer for 30 s. The biosensor was submerged in a 4 μL drop containing 2 μL, 30 μM biotinylated DREAM and 2 μL of fresh dialysis buffer with either 2 mM $CaCl_2$ or 2 mM EGTA for 120 s. A new baseline was acquired for 60 s. The biosensor tip with bound-DREAM was submerged in a 4 μL drop containing 2 μL of 2 μM hexokinase I, and 2 μL of dialysis buffer containing 2 mM $CaCl_2$ or EGTA. The response was recorded for 240 s. The response to dissociation was recorded by submerging the biosensor tip into Buffer A with either 2 mM $CaCl_2$ or EGTA for 240 s. Kinetic constants were derived by using BLItz pro software.

2.9. Measurement of apoptosis in neuroblastoma N1E-115 cells

N1E-115 mouse neuroblastoma cells were grown in DMEM supplemented with 10% FBS and penicillin–streptomycin. Cells in two 24-well plates were transiently transfected using Lipofectamine

with each of the following chimeric plasmids: (1) Sense DREAM cDNA in pcDNA3.1 vector, and hexokinase I cDNA in pCMV6-RFP vector; (2) pcDNA3.1 blank vector and hexokinase I cDNA in a pCMV6-RFP vector, (3) Sense DREAM in pcDNA3.1 vector and a blank cDNA pCMV6-RFP vector, (4) pcDNA3.1 blank cDNA vector and pCMV6-RFP blank cDNA vector. Cells were cultured for 48 h and transferred in equal numbers to a 96-well plate. After 24 h, cells were treated with 5 μg/mL of cisplatin. Caspase-3 activity was measured after 24 h treatment with cisplatin using a Caspase-3 Fluorescence assay kit (Cayman Chemicals, Ann Arbor, MI). Plates were read using a SpectraMax M2 plate reader with excitation and emission wavelengths set to 485 nm and 535 nm, respectively.

2.10. Isolation of mitochondria and assessment of hexokinase-1 bound to mitochondria

N1E-115 neuroblastoma cells were cultured in 60 mm dishes ($n = 18$) and DMEM, 10% FBS. Cells were transfected with chimeric DREAM-pcDNA3.1 plasmid ($n = 9$), or with pcDNA3.1 plasmid ($n = 9$) using Lipofectamine and grown for 24 h post-transfection. Cells were scraped from the dishes and centrifuged at 1000*g* for 5 min. Cell pellets were washed with 1 mL mitochondrial isolation buffer (MIB, 10 mM Tris–HCl, pH 7.4, 0.32 mM sucrose, and 1 mM EDTA). After centrifuging at 1000*g* ~7 volumes MIB was added to the pellet. The pellet was resuspended, and homogenized using a DUALL PTFE pestle and glass tube (Kimble Chase, Vineland NJ). The cell lysate was centrifuged at 1000*g* for five minutes. The resultant supernatant was centrifuged at 18,000*g* for 15 min. 1 mL of MIB was added to the pellet which was centrifuged for 10 min at 18,000*g*. The final mitochondrial pellet was lysed by with H_2O. Protein content was determined by the Bio-Rad protein assay. Thirty μg of protein from each lysate, and 20 ng and 30 ng standards of recombinant hexokinase I were electrophoresed on 10% Bis-Tris SDS–PAGE gels (Life Technologies), and transferred and immunoblotted as described earlier. Hexokinase I bands were imaged and quantitated using the NIH Image program.

3. Results and discussion

3.1. Hexokinase I is captured by a GST-DREAM affinity column in the presence of calcium but not in the presence of EDTA

As shown in Fig. 1, following exposure of rat brain homogenates to GST-DREAM glutathione Sepharose in the presence of Ca^{2+}, a high molecular weight protein band was captured that on subsequent analysis by mass spectrometry was identified as hexokinase I. In the presence of EDTA (and absence of Ca^{2+}) no hexokinase I related peptides were identified on mass spectrometry. The results demonstrated that Ca^{2+} is required for the interaction of DREAM with hexokinase I.

3.2. The interaction of DREAM with hexokinase I is dependent on the presence Ca^{2+}-binding domains of DREAM

As shown in Fig. 2, when rat brain homogenates were bound to a GST-full-length DREAM resin in the presence of Ca^{2+} (Lane 1), bound hexokinase I was readily detected. In the presence of EDTA (Lane 2) bound hexokinase I was not detected. Appropriate controls with GST-resin or resin alone (Lanes 7 and 8, and lanes 9 and 10) showed no hexokinase I binding. To confirm the importance of Ca^{2+}-binding in DREAM-hexokinase I interactions, we examined the binding of hexokinase I derived from rat brain homogenates to a GST-EFMut1234 DREAM resin (Lanes 5 and 6). No binding of hexokinase I was observed, confirming that in the absence of Ca^{2+}-binding to DREAM, interactions with hexokinase I do not occur.

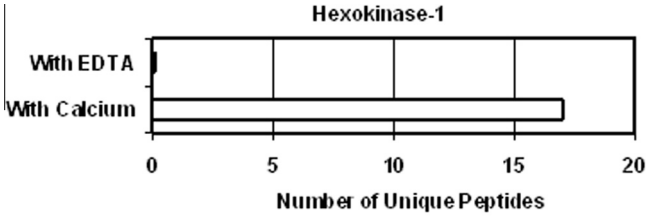

Fig. 1. Total number of unique hexokinase I peptides identified from MS/MS analysis of pull-down experiment with rat brain homogenates applied to a GST-full-length DREAM (amino acids 1–256) resin in the presence or absence of Ca^{2+}.

Further assessment of the influence of Ca^{2+} on DREAM-hexokinase I interactions was performed by bio-layer interferometry. As shown in Table 1, the K_D for the interaction between DREAM-hexokinase I in the presence of Ca^{2+} was 1.57×10^{-6} M whereas in the presence of EGTA the K_D was 5.72×10^{-6} M.

The interaction of DREAM with hexokinase I is dependent on the presence of the NH_2-terminus of DREAM and the presence of Ca^{2+}-binding domains of DREAM: Neuronal calcium-sensor proteins, including DREAM, often bind other proteins via their NH_2-terminal domains. To test whether the NH_2-terminus of DREAM was required for binding to hexokinase I, we examined the interaction of brain-derived hexokinase I with a GST-sFL-DREAM resin. The bound DREAM construct lacks the first 94 residues of DREAM. As noted in Fig. 2, (Lanes 3 and 4), no hexokinase I is bound to the GST-sFL-DREAM resin either in the presence or absence of Ca^{2+}.

3.3. The influence of DREAM on hexokinase I function

As shown in Table 2, the addition of DREAM did not influence the activity of hexokinase I. To assess the influence of DREAM on hexokinase I-modulated apoptosis, we transfected neuroblastoma cells with DREAM and induced apoptosis by the addition of cisplatin and assessed the influence of hexokinase I on this process. Caspase-3 in cisplatin treated, blank vector-transfected neuroblastoma cells (Fig. 3, column 1) is increased by transfection of cells with a DREAM expression plasmid (Fig. 3, column 2, $p < 0.005$). Transfection of neuroblastoma cells with a hexokinase I expression plasmid does not reduce caspase-3 activity when compared to blank vector-transfected cells (Fig. 3, column 4 versus column 1). However, following transfection of neuroblastoma cells with a DREAM expression plasmid, the increased caspase-3 noted in such cells is reduced by co-transfection with a hexokinase I expression plasmid (Fig. 3, column 3 versus column 2, $p < 0.05$).

3.4. DREAM alters hexokinase I localization

To determine how DREAM alters hexokinase I function, we assessed the amount of hexokinase I on mitochondrial membranes

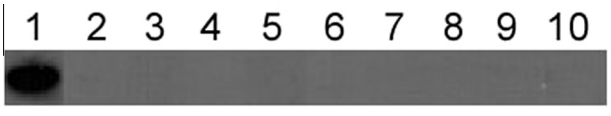

Fig. 2. Western blot analysis of rat brain homogenate proteins bound to various constructs of GST-DREAM immobilized on glutathione resin in the presence or absence of Ca^{2+}. The lane designation are as follows: (1) GST-FL DREAM (1–256), Ca^{2+} present, (2) GST-FL DREAM, EDTA present, (3) GST-sFL DREAM (95–256), Ca^{2+} present, (4) GST-sFL DREAM, EDTA present, (5) GST-EFMut1234 DREAM, Ca^{2+} present, (6) GST-EFMUT1234 DREAM, EDTA present, (7) GST, Ca^{2+} present, (8) GST, EDTA present, (9) Resin, Ca^{2+} present, (10) Resin, EDTA present.

Table 1
The association and dissociation constants of DREAM and hexokinase I as assessed by bio-layer interferometry.

	K_D (M)	k_a (1/Ms)	k_a Error	k_d (1/s)	k_d Error
Ca^{2+} Condition	1.57×10^{-6}	6.28×10^{3}	5.36×10^{2}	9.85×10^{-3}	1.52×10^{-4}
EGTA Condition	5.72×10^{-6}	2.14×10^{3}	1.07×10^{3}	1.23×10^{-2}	3.11×10^{-4}

Table 2
The influence of DREAM on hexokinase I glycolytic activity (mean ± standard deviation).

Buffer alone+Ca^{2+}	DREAM+buffer+Ca^{2+}	Buffer alone+EGTA	DREAM+buffer+EGTA
$n = 63$	$n = 63$	$n = 64$	$n = 64$
8.15 ± 9.11	8.75 ± 9.57	8.35 ± 9.00	9.24 ± 10.22

in the presence and absence of DREAM overexpression. Hexokinase I amount was assessed using immunoblotting and a specific antibody directed against the protein. As shown in Fig. 4, overexpression of DREAM reduced the amount of hexokinase I localized to the surface of mitochondria.

The regulation of apoptosis is important in understanding normal development and the pathogenesis of diseases such as cancer and neurodegenerative diseases [1,3,5–7,25,26]. Apoptosis is controlled by multiple pathways and proteins such as the B-cell lymphoma-2 (Bcl-2) proteins. Many of the effectors of apoptosis alter mitochondrial permeability and the release of cytochrome c, which in turn influences caspase activity [4,26–34]. The VDAC plays a key role in determining mitochondrial permeability [33,35–39]. Its activity is regulated by hexokinase I and II. The binding of hexokinase I and II to the VDAC decreases channel conductance, cytochrome c release and apoptosis [28,40,41]. Overexpression of VDAC1 is noted in Alzheimer's hippocampi, and increases in hippocampal pVDAC1 as well as a decrease in HK1, pAkt and pGSK3 expression are found in a mouse model of Alzheimer's disease [42,43].

EF-hand proteins such as calbindin D_{28K} [44] and DREAM regulate apoptosis [9,10,15,17,19]. DREAM increases the rate of apoptosis in neural cells [15–18] and is expressed in increased amounts in the hippocampus of patients with Alzheimer's disease and in animal models of Alzheimer's disease [15,17,20]. DREAM interacts with the carboxyl-terminal region of presenilin-2 and alters presenilin regulation of γ-secretase activity [9,10]. In addition, DREAM

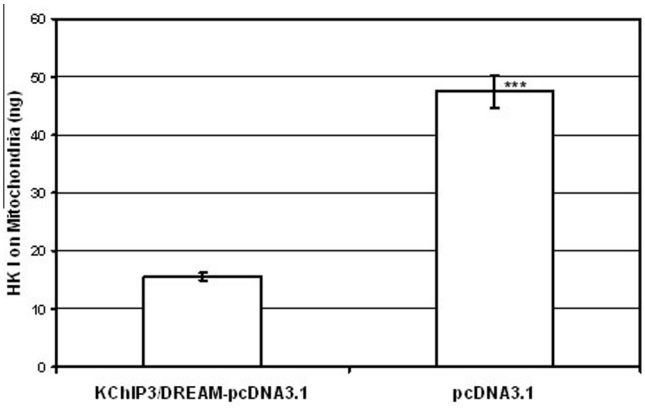

Fig. 4. Hexokinase I bound to isolated mitochondria. Hexokinase I bound (ng) to isolated mitochondria from N1E-115 neuroblastoma cells transfected with either DREAM-pcDNA3.1 or pcDNA3.1. Mean ± standard error of mean. $P < 0.005$, $n = 9$.

modulates intracellular Ca^{2+} concentrations and opposes some of the effects of presenilin on intracellular Ca^{2+} concentrations [19].

The data presented in this report suggests a heretofore unrecognized pathway by which DREAM influences apoptosis through mitochondrial hexokinase I binding and localization. The role of other KChIPs in hexokinase binding was not investigated. It is likely (although not completely definitive) that DREAM binds only hexokinase I, as no peptides from hexokinase II were conclusively identified. Since the amino acid sequences of the hexokinase I and II are 72% identical, and mass spectrometry may not identify segments that are divergent, we cannot be completely sure that DREAM does not bind hexokinase II. The amino acids in hexokinase I required for the interaction with DREAM have not been identified. It is unknown if DREAM displaces hexokinase I from VDAC. The interaction between DREAM and hexokinase I could conceivably be an important target for modulating apoptosis. Targeted down-

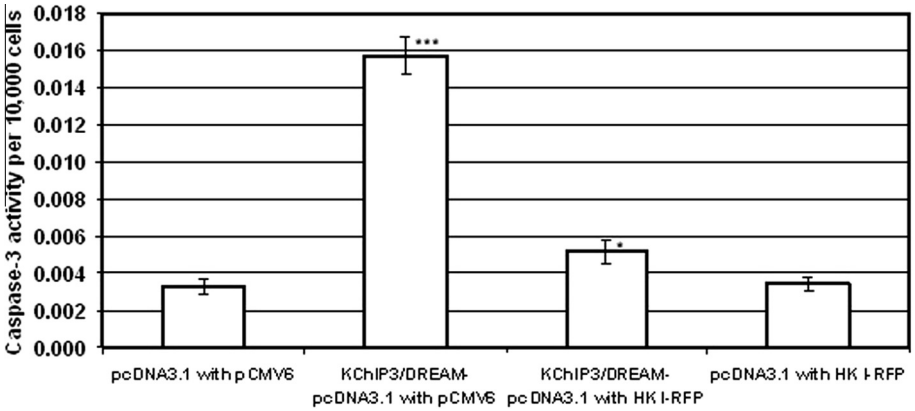

Fig. 3. Caspase-3 assay of cisplatin-induced apoptosis in N1E-115 neuroblastoma cells transfected with DREAM and/or hexokinase I expression plasmids. Column 1. Cells transfected with blank vectors, pcDNA3.1 and pCMV6. Column 2. Cells transfected with sense DREAM-pcDNA3.1 vector and blank pCMV6 vector. Column 3. Cells transfected with DREAM-pcDNA3.1 vector plus hexokinase I-RFP vector. Column 4. Cells transfected with hexokinase I-RFP vector and pcDNA3.1 blank vector. The cells were treated with 5 μg/ml cisplatin for 24 h. Number of unique observations = 5. $^{*}P < 0.05$, $^{***}P < 0.005$.

regulation of DREAM may allow binding of HK1 to VDAC preventing apoptosis and neurodegeneration.

4. Conclusion

DREAM alters apoptosis in neural cells by binding hexokinase I, and reducing mitochondrial hexokinase I localization.

Acknowledgments

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