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In vitro ischemia decreases histone H4K16 acetylation in neural cells

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ABSTRACT

Inhibitors of histone deacetylases are frequently used against ischemia-induced injury, but the specific mechanisms of their action are poorly understood. Here, we report that following a 5–7-h oxygen–glucose deprivation (OGD) acetylation of histone H4 at residue K16 (H4K16Ac) decreases by 40–80% in both PC12 cells and primary neurons. This effect can be reverted by treatment with _trichostatin A, or by supplementation with acetyl-CoA. A decrease in H4K16Ac levels can affect the expression of mitochondrial uncoupling protein 2 (UCP2), huntingtin-interacting protein 1 (HIP1) and Notch-pathway genes in a cell-specific manner. Thus, H4K16 acetylation is important for responses to ischemia and cell energy stress, and depends on both cytosolic and mitochondrial acetyl-CoA.

1. Introduction

Ischemia is a common patho-physiological condition characterized by a lack of blood supply and temporary energy deprivation in brain or myocardial tissue, and results in stroke and infarction [1–4]. This is investigated with different in vivo and in vitro models [5–7]. The popular in vitro model of cerebral ischemia is "oxygen and glucose deprivation" (OGD), which impacts the function of cells in white and gray matter in the brain [1,8]. Sustained ischemia is generally damaging to cells; however, pre-conditioning with short pulses of hypoxia followed by re-perfusion with oxygen-saturated nutrient-rich media has been shown to improve cell survival during subsequent stroke conditions [8,9]. Cell energy stress and ischemia–reperfusion processes, studied at the molecular level in various models and cell types (neurons, astrocytes, endothelial cells), also revealed major roles of hypoxia-inducible factors in ischemic tolerance [1,10–13].

Epigenetic factors represent another mode of regulation of ischemic tolerance [4,14–16]. Thus, hypoxic and anoxic conditions affect histone methylation, while histone deacetylase inhibitors (HDACi) such as valproic acid or trichostain A show neuroprotective effects during ischemia [17,18]. A number of chromatin-remodeling complexes, such as SWI/SNF, are involved in cellular responses to ischemia, with the hypoxia-inducible factor (HIF) pathway being one of the most important and well studied [19–21]. Availability of metabolic substrates is also important: reversible acetylation of histones and other proteins depends on the levels of NAD^+ and acetyl-CoA [22,23].

The cellular acetyl-CoA pool is distributed between the cytosol and mitochondria. Cytosolic acetyl-CoA drives acetylation of numerous cytosolic and nuclear proteins, while the mitochondrial pool is essential for the function of the electron transport chain [1]. However, recently it was shown that the mitochondrial pool of acetyl-CoA can also contribute (up to 50%) to the acetylation of nuclear histones [24,25]. This suggests that under ischemia, when the pyruvate dehydrogenase complex is inhibited, mitochondria can act as cellular stress "sensors" epigenetically regulating gene expression via histone acetylation.

Among the multiple sites of histone acetylation, the K16 residue in histone H4 (H4K16Ac) is considered to be one of the versatile epigenetic marks. It regulates chromatin remodeling and global activation of gene expression [26], and is often coordinated with histone H3K4 mono-, di- and trimethylation, and cell cycle progression [27,28]. Abnormal levels of H4K16Ac lead to defects in

Abbreviations: ACA, acetyl-CoA; BA, sodium butyrate; DCA, sodium dichloroacetate; GD, glucose deprivation; H4K16Ac, histone H4 acetylated at residue K16; HAT, histone acetyltransferase; HDAC, histone deacetylase; HDACi, inhibitor of histone deacetylase; HIF, hypoxia inducible factor; OD, oxygen deprivation; OGD, oxygen and glucose deprivation; RT-PCR, reverse transcription-polymerase chain reaction; TSA, trichostatin A

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DNA repair, RNA polymerase II-mediated transcription, cell cycle arrest, altered autophagy and embryonic lethality [29–33]. In mammals this modification is regulated by histone acetyltransferases KAT8 (MYST1, MOF) [29], KAT5 (TIP60) [34]and KAT2A (GCN5) [35], and by histone deacetylases such as Sirt1 and HDAC1 [36,37]. These members of distinct multi-protein nuclear complexes [27,37,38] are often cross-regulated [39] and can reversibly acetylate many important proteins including p53, PGC-1α and others [40,41].

Here, we hypothesized that sustained OGD affects H4K16Ac which in turn contributes to ischemic tolerance, and studied this with two types of neural cells: PC12 rat pheochromocytoma cells and mixed culture of primary neurons. We found that H4K16Ac was markedly reduced over 6 h of OGD, and almost fully restored by subsequent re-perfusion in PC12 cells. We studied the mechanism of such decrease and found that it is due to the function of histone deacetylases. Ischemia-dependent decrease of H4K16Ac affects gene expression in a cell-specific manner, and this can be exploited in development of specific therapies for stroke. Preliminary data from this work were reported in [42].

2. Methods

2.1. Materials

CellTiter-Glo reagent, CellTox Green viability assay kit, ImProm-II reverse transcriptase, PCR master mix, ribonuclease inhibitor and SV Total RNA Isolation system were from Promega (MyBio, Kilkenny, Ireland). Nucleo-spin II RNA extraction kit was from Macherey–Nagel (Fisher Scientific, Dublin, Ireland). Sodium dichloroacetate (DCA) was from Tocris Bioscience (Abingdon, UK). Rabbit anti-histone H4 (04-858), H4K16Ac (07-329) antibodies were from Millipore (Cork, Ireland). Goat anti-MOF, TIP60 and VDAC antibodies were from Santa Cruz Biotechnology (Heidelberg, Germany). Mouse monoclonal anti-GCN5/KAT2A, anti-Sirt1, rabbit polyclonal anti-KDM1/LSD1, anti-histone H3 (tri-methyl K4) and anti-pyruvate dehydrogenase kinase 1 antibodies were from Abcam (Cambridge, UK). Anti-acetylated-lysine mouse mAb (Ac-K-103) antibody was from Cell Signaling Technology (Dublin, Ireland), anti-PGC-1α rabbit antibody was from Calbiochem-Millipore (Cork, Ireland). Anti-HIF-2α goat antibody (AF2886) was from R&D systems (Minneapolis, USA). Secondary IRdye 800CW antibodies were from LI-COR Biosciences (UK). SafeView DNA stain was from NBS Biologicals (UK). Pre-cast polyacrylamide gels, and electrophoresis running and transfer buffers were from Genscript (Piscataway, NJ, USA). ECL Plus and Prime western blotting reagents were from GE Healthcare Life Sciences (Cork, Ireland). Anti-α-tubulin and acetyl-α-tubulin antibodies, horseradish peroxidase-conjugated secondary anti-rabbit (RG-96), goat/sheep (GT-34) and mouse (A9917) antibodies, (+/−)-carnitine, DL-acetylcarnitine, protease inhibitor cocktail, sodium butyrate, valproic acid, trichostatin A and all the other reagents were from Sigma–Aldrich (Dublin, Ireland). Oligonucleotide primers were synthesized by Sigma–Aldrich (Dublin, Ireland).

2.2. Cell culture

PC12 rat pheochromocytoma cells were cultured in suspension in RPMI1640 medium supplemented with 10% horse serum and 5% fetal bovine serum as described before [43]. For oxygen and glucose deprivation (OGD), cells were seeded in 25 cm^2 flasks (low attachment surface, Corning) at 2–2.5 $*$ 10^6/flask. Next day they were collected by centrifugation (200×*g*, 5 min), quickly resuspended in OGD medium (phenol red- and glucose-free DMEM, supplemented with 1 mM sodium pyruvate, 2 mM glutamine, 10 mM HEPES-Na, pH 7.2) in 25 cm^2 flasks, placed at 0% O_2 (verified with "Optech Platinum" –O_2 sensor, Mocon, USA) [44] in a modular incubator chamber (Billups-Rothenberg, Inc, USA) for 0–9 h, followed by histone, total protein or RNA extraction.

For assessment of cell viability by measuring total ATP, cells were seeded onto collagen IV-coated StripWell plates (Corning, USA) at a density of 150 000/well, allowed to attach (1.5 h), exposed to OGD and re-perfusion, and then lysed using CellTiter-Glo reagent as per manufacturer's instructions and measured on a microplate reader (total luminescence). For assessment of cell viability with the CellTox Green kit, cells were seeded and treated similarly, and then analyzed using an end-point method as per manufacturer's instructions on a fluorescence plate reader (485/535 nm filters, top reading). The control (untreated) cells were used as 100% viable and lysed as 0%.

Results of cell viability assays were normalized by extracting total protein with PEB buffer (50 mM HEPES-Na, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Igepal CA630, protease inhibitor cocktail Sigma P2714) and analysis with a BCA Protein assay kit (Pierce, Thermo Fisher, Ireland). Control experiments were performed in medium supplemented with D(+)-glucose (10 mM), serum or drugs, as described in the "Results" section. For re-perfusion, samples exposed to OGD were pelleted, resuspended in complete growth media, incubated at ambient O_2 for further 18 h and then analyzed.

The mixed culture of embryonic rat E18 cortical neurons was obtained as described previously [45]. All the procedures with animals were performed under a license issued by the Irish Government Department of Health and Children (Ireland) and in accordance with the Directive 2010/63/EU adopted by the European Parliament and the Council of the European Union. Briefly, cells were seeded on poly-D-lysine-coated 25 cm^2 flasks (10 $*$ 10^6 cells/flask) in DMEM-F12 Ham medium supplemented with 2% B27, 1% fetal bovine serum and 1% penicillin–streptomycin, cultured for 7 days at ambient O_2, and then exposed to OGD as above in media supplemented with 2% B27.

2.3. Protein extraction and Western blotting

Histones were extracted as described before [46]. Extracts were cleared by centrifugation (600×*g*, 10 min) and total proteins were quantified by the Bradford method. Samples normalized for total protein were mixed with 5× Sample Laemmli Buffer, incubated for 10 min at room temperature, and analyzed by western blotting as described before [43], using either chemiluminescent (ECL Plus) or fluorescently labeled (IRdye 800CW) secondary antibodies and detection.

For total protein extraction, 5 $*$ 10^6 cells exposed to OGD, were supplemented with 5 mM sodium butyrate (BA), washed in ice-cold PBS supplemented with 5 mM sodium butyrate (BA), lysed in 500 µl of PEB buffer (30 min, 4 °C), pelleted (12 000 g, 15 min). Soluble extracts were quantified by BCA protein assay kit.

2.4. RNA extraction and RT-PCR

4–5 $*$ 10^6 cells exposed to OGD were collected and washed in PBS, supplemented with 5 mM BA as above, washed in 300 µl of RNA later reagent and stored at −18 °C prior the extraction. Total RNA was isolated using SV Total RNA isolation system or Nucleo-Spin II RNA extraction kit, accordingly to manufacturer's instructions. 1–2 µg of total RNA were annealed with 500 ng oligo-$(dT)_{15}$ (70 °C for 5 min, 4 °C for 5 min), reverse transcribed using ImProm-II reverse transcriptase, and analyzed by PCR (25–30 cycles). Oligonucleotide primer sequences are provided in Supplementary Table 1.