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The sigma-1 receptor-zinc finger protein 179 pathway protects against hydrogen peroxide-induced cell injury

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ABSTRACT

The accumulation of reactive oxygen species (ROS) have implicated the pathogenesis of several human diseases including neurodegenerative disorders, stroke, and traumatic brain injury, hence protecting neurons against ROS is very important. In this study, we focused on sigma-1 receptor (Sig-1R), a chaperone at endoplasmic reticulum, and investigated its protective functions. Using hydrogen peroxide (H_2O_2)-induced ROS accumulation model, we verified that apoptosis-signaling pathways were elicited by H_2O_2 treatment. However, the Sig-1R agonists, dehydroepiandrosterone (DHEA) and DHEA sulfate (DHEAS), reduced the activation of apoptotic pathways significantly. By performing protein–protein interaction assays and shRNA knockdown of Sig-1R, we identified the brain Zinc finger protein 179 (Znf179) as a downstream target of Sig-1R regulation. The neuroprotective effect of Znf179 overexpression was similar to that of DHEAS treatment, and likely mediated by affecting the levels of antioxidant enzymes. We also quantified the levels of peroxiredoxin 3 (Prx3) and superoxide dismutase 2 (SOD2) in the hippocampi of wild-type and Znf179 knockout mice, and found both enzymes to be reduced in the knockout versus the wild-type mice. In summary, these results reveal that Znf179 plays a novel role in neuroprotection, and Sig-1R agonists may be therapeutic candidates to prevent ROS-induced damage in neurodegenerative and neurotraumatic diseases.

1. Introduction

Reactive oxygen species (ROS) including free radicals, such as superoxide anion ($\cdot O_2$-) and hydroxyl radical (OH), as well as non-radical oxidants, such as singlet oxygen ($^1O_2$) and hydrogen peroxide (H_2O_2), are produced as by-products of cellular metabolism, majorly in the mitochondria (Giorgio et al., 2007). In normal cellular processes, ROS are regulated at relatively low steady-state levels (background levels) by endogenous antioxidant enzymes. However, many neurodegenerative diseases, including Alzheimer's (AD), Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS), Huntington's disease (HD), and neurotraumatic diseases, including stoke, traumatic brain injury (TBI), show a reduction in antioxidant capacity and/or an increase in ROS production (Gilgun-Sherki et al., 2001; Liu et al., 2015; Lin and Beal, 2006; Xiong et al., 2013). They may result from impaired mitochondrial function or environmental stressors including sugar/oxygen deprivation in ischemia, cytokine release in inflammation, excitatory neurotransmitter release, and metabolic depression (Lin and Beal, 2006; Xiong et al., 2013; Hetz and Mollereau, 2014). Left unchecked, the accumulated ROS like the hydroxyl free radical will damage DNA and induce apoptosis. Therefore, preventing ROS accumulation or enzymatically eliminating them is an important subject in neurological disorders

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therapy.

The sigma-1 receptor (Sig-1R) is widely expressed in the endoplasmic reticulum (ER) and mitochondrial and plasma membranes of neurons throughout the central nervous system (Kourrich et al., 2012). It binds certain neuroleptic drugs, psychotropic drugs, and steroid hormones (Kourrich et al., 2012; Hayashi and Su, 2001). These drugs regulate the ability of Sig-1R to associate with other ER protein partners, including an inositol 1-4-5-triphosphate receptor (IP3R), the potassium voltage-gated channel Kv1.2, and Binding immunoglobulin protein (BiP) (Ishikawa, 2010; Kourrich et al., 2013). Activation of Sig-1R appears to provide neuroprotection and neurorestoration in cellular and animal models of brain ischemia, and in neurodegenerative diseases such as AD, PD, and ALS (Nguyen et al., 2015; Ruscher and Wieloch, 2015).

Dehydroepiandrosterone (DHEA) and its sulfated analog (DHEAS) are steroid hormone agonists of Sig-1R (Su et al., 1988; Monnet et al., 1995; Maurice et al., 1996). DHEA/DHEAS are secreted by the adrenal gland and brain, and can prevent excitatory amino acid-induced neurotoxic actions in primary hippocampal neurons and retinal cells, reduce apoptosis in a model of serum-free PC12 cell death, and attenuate the lesion area of focal brain injury (Charalampopoulos et al., 2004; Kokona et al., 2012). Additionally, some studies indicate that the two steroid hormones perform antioxidant functions in age-related neurodegenerative disorders and cardiovascular disease by increasing the expression of antioxidant proteins such as superoxide dismutases (SODs) (Grimm et al., 2014; Camporez et al., 2011). However, the relationship between DHEA/DHEAS-mediated antioxidant protein expression and Sig-1R signaling, and whether either activity is applicable in neurological disorder therapy, is still unknown.

The zinc finger protein 179 (Znf179), also known as RING finger protein 112 (Rnf112), is predominantly expressed in the central nervous system (Kimura et al., 1997), and is known to be active in nervous system development during embryogenesis (Pao et al., 2011). Although the roles of Znf179 post-embryogenesis still remain unclear, it is a downstream target of Sig-1R, and could theoretically mediate the neuroprotective effects of DHEA/DHEAS. This study evaluates the functional effects of both Sig-1R and Znf179 in relation to ROS-induced damage.

2. Materials and methods

2.1. Cells

Mouse neuroblastoma Neuro-2a (N2a) cells (ATCC) were cultured in minimum essential medium Eagle (MEM, Invitrogen) containing 10% fetal bovine serum (FBS), 100 μg/ml streptomycin sulfate, and 100 U/ml penicillin-G sodium at 37 °C and 5% CO2. N2a cells were differentiated after serum withdrawal by incubation in MEM/BSA medium (MEM supplemented with 0.1% bovine serum albumin and without FBS) (Evangelopoulos et al., 2005). The 90% confluent cells were treated with DHEA (1 μM or 10 μM, Sigma−Aldrich), DHEAS (1 μM or 10 μM, Sigma−Aldrich), or BD1063 (1 μM, Tocris Bioscience) for 30 min before H2O2 treatment. Transfection of cells with protein-expressing vectors or shRNA plasmids was performed by using Lipofectamine 2000 according to the manufacturer's protocol (Invitrogen). Each transfection experiment was performed three times and each sample in each experiment was prepared in duplicate.

2.2. Western blot analysis

Protein samples were separated via sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred onto a PVDF membrane (Bio-Rad Laboratories). Subsequently, the membrane was blocked with 5% nonfat milk in TBST buffer (10 mM Tris. pH 8.0, 150 mM NaCl, and 0.5% Tween 20) for 1 h and incubated with primary antibodies: Anti-Sig-1R (1:500), anti-emerin (1:2000), anti-Nesprin1 (1:500), anti-Sun2 (1:500), anti-(activating transcription factor6) ATF6 (1:500) antibodies from Santa Cruz Biotechnology, anti-p53 (1:3000), anti-phospho-p53 (Ser15) (1:3000), anti-p38 (1:2000), anti-phospho-p38 (Thr180/Tyr182) (1:1000), anti-JNK (1:2000), anti-phospho-JNK (Thr183/Tyr185) (1:500), anti-ERK (1:3000), anti-phospho-ERK (Thr202/Tyr204) (1:3000), anti-(poly (ADP-ribose) polymerase) PARP (1:2000), anti-TNF-alpha (1:1000) antibodies from Cell Signaling Technology, anti-SOD1 (1:3000), anti-SOD2 (1:3000), anti-Prx3 (1:1000), anti-caspase 3 (1:500) antibodies from GeneTex, anti-actin (1:3000), anti-Sp3 (1:1000), HDAC1 (1:2000), anti-HDAC2 (1:2000) antibodies from Millipore, anti-MeCP2 (1:1000) antibody from Abcam, anti-(binding immunoglobulin protein) BiP (1:3000) from BD biosciences, and anti-Znf179 (1:1000) antibody (Pao et al., 2011; Lin et al., 2013), for 2 h at room temperature. After incubation with primary antibodies, the membranes were washed three times (5 min each) with TBST buffer, and then incubated with a 1:3000 dilution of horseradish peroxidase-conjugated anti-mouse or anti-rabbit antibodies (Santa Cruz Biotechnology) for 1 h at room temperature. Finally, the membranes were washed again and the peroxidase was developed by chemiluminescence using Amersham Hyperfilm ECL (GE Healthcare).

2.3. His-pull-down assay

His-Sig-1R expressing cells were lysed by using modified RIPA buffer (50 mM Tris, pH 7.8, 150 mM NaCl, 5 mM EDTA, 0.5% Triton-X100, 0.1% Nonidet P-40, and protease inhibitors) contained 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml leupeptin, 2 μg/ml aprotinin, and 1 mM imidazole. The cell lysates were then added with 30 μl of HisPur Ni-NTA Superflow Agarose (Thermo Scientific) and incubated at 4 °C with shaking for 2 h. The beads were subsequently washed three times in modified RIPA buffer containing 10 mM imidazole. Bound proteins were eluted by using electrophoresis sample buffer and analyzed by western blot using specific antibodies, such as ER proteins (binding immunoglobulin protein (BiP), activating transcription factor (ATF) 6, etc.), nuclear envelope proteins (emerin, lamin B, lamin-B receptor, lamin A/C, Nesprin1, Sun2, etc.), transcription factors (Sp3, MeCP2, barrier-to-autointegration factor (BAF), etc.), and cytoplasmic−nuclear proteins (Histone deacetylases (HDACs), Znf179, extracellular signal-regulated kinases (ERK) 1 and 2, etc.).

2.4. Immunoprecipitation

Cells (1×10^7) were washed with PBS, and the cellular lysates were prepared by using modified RIPA buffer. Rabbit polyclonal anti-Znf179 antibodies or control rabbit IgG were then added (1:500) to the lysates and incubated at 4 °C with rotation. After 2 h, protein-A/G agarose beads (30 μl, Santa Cruz Biotechnology) were also added to the mixture and further incubated for 1 h. The protein beads were washed three times with modified RIPA buffer. Bound proteins were eluted by using electrophoresis sample buffer.

2.5. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay

N2a cells were plated onto 24-well culture plates at an initial density of 1×10^5 cells/well. After the induction of neuronal differentiation, cells were treated with different doses of H_2O_2 for 24 h or were treated with 50 μM H_2O_2 for different time intervals. Subsequently, fresh medium containing MTT (3-(4,5-