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The melanocortin ACTH 1-39 promotes protection of oligodendrocytes by astroglia☆

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ABSTRACT

Damage to myelin and oligodendroglia (OL) in multiple sclerosis (MS) results from a wide array of mechanisms including excitotoxicity, neuroinflammation and oxidative stress. We previously showed that ACTH 1-39, a melanocortin, protects OL in mixed glial cultures and enriched OL cultures, inhibiting OL death induced by staurosporine, ionotropic glutamate receptors, quinolinic acid or reactive oxygen species (ROS), but not nitric oxide (NO) or kynurenic acid. OL express melanocortin receptor 4 (MC4R), suggesting a direct protective effect of ACTH 1-39 on OL. However, these results do not rule out the possibility that astroglia (AS) or microglia (MG) also play roles in protection. To investigate this possibility, we prepared conditioned medium (CM) from AS and MG treated with ACTH, then assessed the protective effects of the CM on OL. CM from AS treated with ACTH protected OL from glutamate, NMDA, AMPA, quinolinic acid and ROS but not from kainate, staurosporine, NO or kynurenic acid. CM from MG treated with ACTH did not protect from any of these molecules, nor did CM from AS or MG not treated with ACTH. While protection of OL by ACTH from several toxic molecules involves direct effects on OL, ACTH can also stimulate AS to produce mediators that protect against some molecules but not others. Thus the cellular mechanisms underlying the protective effects of ACTH for OL are complex, varying with the toxic molecules.

1. Introduction

Damage to myelin, oligodendroglia (OL) and neurons in multiple sclerosis (MS) occurs by multiple mechanisms ([19], review). We have established an in vitro approach [3,4] to investigate the efficacy of potential therapeutic agents in preventing death of OL, OL progenitors (OPC) and neurons from cytotoxic agents involved in several of these mechanisms, including excitotoxicity, inflammation-related signals, apoptotic signals and reactive oxygen species (ROS) [5,6,30,31]. One of these potentially protective agents, ACTH 1-39, has been used to treat MS relapses, and was thought to act primarily on melanocortin receptors in the adrenals to stimulate release of corticosteroids. However, neurons, astrocytes (AS), microglia (MG) and oligodendroglia (OL) all express melanocortin receptors [1,6,10,12,42], raising the possibility that melanocortins can act directly on these cells.

We found that ACTH 1-39, a melanocortin, protected OL in mixed glial cultures, inhibiting OL death induced by staurosporine, glutamatergic ionotropic receptors, quinolinic acid (QA) or ROS, but not nitric oxide (NO) or kynurenic acid [6]. We also showed that ACTH 1-39 protected OL in cultures enriched (85–90%) for OL. The OL express melanocortin receptor 4 (MC4R), suggesting a direct effect. While these results support a direct effect of ACTH on OL, they do not rule out the possibility that AS or MG could also play roles in ACTH-mediated protection. To investigate this, we prepared conditioned medium (CM) from AS and MG treated with ACTH 1-39, then assessed the protective effects of the CM on OL treated with the panel of cytotoxic agents.

2. Materials and methods

2.1. Reagents

The ionotropic glutamate receptor ligand α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) was purchased from Tocris (Ellisville MO); the NO donors NOC-12 and NOC-18 were from Calbiochem (LaJolla CA). The other reagents, including adrenocorticotropic hormone 1–39 (ACTH 1-39) and *N*-methyl-D-aspartic acid (NMDA), were from Sigma (St. Louis MO). Cell types were characterized using the following antibodies to phenotypic markers: platelet derived growth factor receptor alpha (PGDFRα) for OPC [36] from Santa Cruz Biotechnology, Dallas TX; galactocerebroside for OL, monoclonal antibody generated from O1 hybridoma cells [43]; anti-glial fibrillary acidic protein (GFAP) (Chemicon International Inc., Temecula CA, M#B360) for AS [37] and the lectin IB4 (Sigma, L9381) for MG [34].

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2.2. Preparation of cultures enriched in oligodendroglia

Cultures were prepared from brains of 2–3 day old rats as previously described [18,29]. For each of the experiments, three forebrains were carried through the dissociation procedure, suspended in 16 ml of Dulbecco's minimal essential medium (DMEM) containing 10% newborn calf serum (NCS) and plated in two T75 flasks. For cultures highly enriched in mature OL, the flasks containing the glial bed layers were subjected to shakeoff every 5–7 days. Cells from the two flasks after the 4th and 5th shakeoffs were plated on a 100 mm uncoated plastic dish for 1 h, and the unattached cells replated on plastic for an additional hour to remove MG. The unattached cells were pelleted, resuspended in 2.5 ml of OL defined medium [6], then 50 μl aliquots were plated on 50 poly-D-lysine coated coverslips and allowed to attach overnight. OL defined medium with 1% newborn calf serum (NCS) was added for the next 24 h, then OL defined medium with 2% NCS [18]. After 6–8 days in OL defined medium with 2% NCS, the enriched OL cultures were treated with ACTH 1-39, the cytotoxic reagents and/or conditioned medium. Untreated cultures with defined medium with 2% NCS served as control. Some of the enriched OL cultures were assessed for composition as described previously [6], using immunocytochemistry with antibodies to cell type specific markers, including galactolipids for OL, glial fibrillary protein for AS, and A2B5 for OPC. Binding of the lectin IB4 was used to detect MG. As found previously in our laboratory, the cultures contained on average 88% differentiated OL, 3% AS, 8% MG and less than 1% OPC.

2.3. Preparation of astroglial cultures

OL and MG were removed from mixed glial cultures [29] by shake-off from one T75 flask (cells from 1½ forebrains, as above), leaving behind the bed layer of highly enriched AS. The AS cultures were treated with 0.05% trypsin, pelleted, washed with DMEM containing 10% NCS, then plated on poly-lysine coated flasks. The AS cultures were maintained in 10% NCS in DMEM, then used after 1 week to produce AS conditioned medium (CM).

2.4. Preparation of microglial cultures

Cells obtained from shake-off of two T75 flasks of mixed glial cultures (16 ml of 10% NCS in DMEM, cells from 3 rat forebrains, as above) were plated on uncoated plastic dishes for 2 h and non-adherent cells removed. The adherent cells were maintained in 10% NCS in DMEM for one day, then used to prepare CM. The cells were 87% MG based on binding of the lectin IB4.

2.5. Preparation of conditioned medium

Purified AS or MG were incubated for 24 h with either DMEM or with DMEM containing 200 nM ACTH 1-39. Supernatants were removed, and cultures were washed with DMEM to remove ACTH in the medium; DMEM was added for an additional 24 h, then collected to give conditioned medium (CM) from AS or MG pretreated with ACTH, designated as AS CM ACTH or MG CM ACTH, respectively. Controls consisted of medium from AS and MG treated as above except not exposed to ACTH, AS CM and MG CM, respectively. Supernatants were stored at −20 °C until used.

2.6. Cell treatment and measurement of cell death

For analysis of the effects of ACTH 1-39 or CM on OL death, purified OL cultures were incubated with ACTH 1-39 at 200 nM or the various CM for 30 min before addition of the toxic agents. Cell death was assessed after 1 day using trypan blue uptake as the indicator of cell death as described in our previous studies [3,4]. Trypan blue is considered a preferred method for measurement of total cell death [35] compared to terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL), which measures only apoptosis, or live/dead fluorescent assays, which may not detect permeable dead cells with degraded DNA, thus underestimating cell death [46]. Differentiated OL were identified by their characteristic morphology, that is, rounded or oval birefringent cells with multiple lacy branching processes, and in some cases by immunostaining with antibodies to galactolipids.

The % of trypan blue + OL was determined by counting between 100 and 200 OL in 4–6 random fields on two coverslips per experiment with 3 or more independent experiments for each condition. Values for duplicate coverslips agreed within 10% of one another.

The concentration of ACTH was selected based on the concentration giving optimal protection against the toxic agents [6]. Concentrations of the cytotoxic agents were selected to give 60–75% OL death after 1 day, with no death of AS or MG ([6]; see legends in graphs). All experiments were repeated three times with duplicate coverslips in each experiment. Results were analyzed by one-way ANOVA with Tukey post-test ($p < 0.05$).

3. Results

3.1. Astrocyte conditioned medium and microglia conditioned medium do not affect oligodendroglial viability at 24 h

We first prepared CM from untreated AS cultures and from AS cultures treated with 200 nM ACTH 1-39 for 24 h, washed to remove ACTH 1-39, then incubated for another 24 h in DMEM as detailed in Methods. In initial experiments, we found no difference in OL viability in the presence of OL defined medium with 2% NCS or AS CM (prepared in DMEM with no serum). After 24 h, OL death under each condition varied between 1 and 4% (data not shown). Similar results for OL viability were obtained with MG CM. In subsequent experiments, controls in each experiment consisted of OL in defined medium with 2% NCS.

3.2. Astrocytes treated with ACTH secrete factors that protect OL from cytotoxic insults

We previously showed that ACTH can protect OL from a variety of cytotoxic insults [6]. To investigate whether ACTH stimulated AS to produce factors protective to OL, we compared AS CM ACTH to ACTH for protection against excitotoxic, inflammation-related, oxidative and nitrosylation mediated OL death.

With regard to excitoxicity, glutamate can act via ionotropic glutamate receptors on OL to induce cell death. As shown in Fig. 1, in agreement with our previous results [6], ACTH 1-39 at 200 nM protected OL from killing by 100 μM glutamate, reducing OL death at 24 h from 52% to 28% ($p < 0.05$). Glutamate was slightly more toxic to OL in the presence of AS CM compared to control medium, although the differences were not significant (58% vs. 52%, AS CM + glutamate vs. control medium + glutamate, ns). However, AS CM ACTH (from AS cultures pretreated for 24 h with ACTH then washed to remove ACTH before collection of CM) protected OL against glutamate compared to AS CM itself (46% vs. 59%, AS CM ACTH + glutamate vs. AS CM + glutamate, $p < 0.05$). Under these culture conditions, ACTH at 200 nM was more effective than AS CM ACTH in protecting OL from glutamate (28% vs. 46%, ACTH + glutamate vs. AS CM ACTH + glutamate, $p < 0.05$).

When OL death was induced with the ionotropic glutamate ligand NMDA at 2 mM, both ACTH 1–39 in control medium and AS CM ACTH were protective under these conditions (Fig. 1). In these experiments, ACTH at 200 nM was slightly more effective than AS CM ACTH in protecting OL from NMDA (30% vs. 37%, ACTH + NMDA vs. AS CM ACTH + NMDA, $p < 0.05$) compared to control medium + NMDA (55% OL death). Again, AS CM from AS not pretreated with ACTH was not protective for OL treated with NMDA, similar to control medium (57% vs. 55%, AS CM + NMDA vs. control medium + NMDA, ns).