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Enhancing the interaction between annexin-1 and formyl peptide receptors regulates microglial activation to protect neurons from ischemia-like injury

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ABSTRACT

As the immune cells of the brain, microglia are crucial for the maintenance of brain function. The aims of the present study were to determine whether and how annexin-1 is able to affect microglial phenotype and migration in the lesion microenvironment. In the current experiment, we enhanced the interaction between annexin-1 and formyl peptide receptors in microglia and analyzed the function. We found that annexin-1 could polarize microglia to a beneficial phenotype and promote microglial migration to protect neurons from ischemia-like injury, and the annexin-1-mediated neuroprotective effect was dependent on the release of glutamate and ATP from the injured neurons.

1. Introduction

Microglia are the resident immune cells of the central nervous system that continually monitor the brain microenvironment (Ransohoff and Perry, 2009). In the event of a brain injury, such as that observed during a neurodegenerative disease, cerebral ischemia or traumatic injury, the continued presence of "danger" signals resulting in the subsequent production of proinflammatory biomolecules, reactive oxygen and nitrogen species, prostaglandins and other factors, which exacerbate CNS insults, activates microglia (Nakajima and Kohsaka, 2004; Mitrasinovic et al., 2005). In addition, activated microglia also release a range of trophic cytokines, such as glutamate transporters, antioxidants and anti-inflammatory cytokines. The microglia migrate to the affected site to "rescue" mildly injured cells or to "destroy" severely damaged cells that are beyond rescue and to clear cellular debris and toxic substances by phagocytosis (Koizumi et al., 2007; Lai and Todd, 2008). Because of this dual nature, two new classifications of microglia have entered into the literature in recent years, described as the classically activated pro-inflammatory (M1) and alternatively activated anti-inflammatory (M2) phenotypes. However, whether microglia exert a neurotoxic or neuroprotective effect following CNS injury is still controversial (Danton and Dietrich, 2003; Nakajima and Kohsaka, 2004; Imai et al., 2007).

Annexin-1, a 37 kDa member of the annexin superfamily of proteins, previously known as lipocortin1, has potent anti-inflammatory properties (Perretti and D'Acquisto, 2009). Since its discovery, annexin-1 has been shown to regulate diverse cellular functions in a variety of cell types. Intracellularly, annexin-1 plays a role in differentiation (Solito et al., 1998), proliferation (de Coupade et al., 2000), and plasma membrane repair (McNeil et al., 2006), as well as in eicosanoid production (Flower, 1988), and apoptosis (McKanna, 1995; Solito et al., 1998; Vago et al., 2012). There is also overwhelming evidence of a role for extracellular annexin-1 in several anti-inflammatory processes (John et al., 2007; Vago et al., 2012), including the regulation of neutrophil migration and macrophage phagocytosis (Lim et al., 1998; Fan et al., 2004). In particular, annexin-1 promotes the phagocytic removal of apoptotic leukocytes by macrophages without the production of inflammatory mediators and therefore without the escalation of the inflammatory cascade (Morimoto et al., 2006; Li et al., 2011). Although its function in the central nervous system is still elusive, enhanced expression of annexin-1 has been reported at sites of damage in the brains of patients suffering from multiple sclerosis (Probst-Cousin et al., 2002), Parkinson's disease (Knott et al., 2000), and Alzheimer's disease (McArthur et al., 2010).

In the present study, we investigated the signaling mechanism by which the activation of microglial nFPRs might regulate microglial phenotype and migration. We used rat cortical neurons and microglia

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to address both neuron-to-microglia and microglia-to-neuron communications. Furthermore, we used the BV-2 cell lines to clarify the molecular mechanism involved in the interaction between annexin-1 and nFPRs. Our results show that nFPR signaling plays a cytoprotective role in cortical neurons following oxygen glucose deprivation (OGD) and that enhancing the annexin-1–nFPR interaction significantly favors the switch of microglia from the inflammatory M1 type to the anti-inflammatory M2 type. Finally, nFPR-mediated signaling was also shown to be involved in microglial migration. Taken together, our results provide a new view on brain protection from the perspective of microglia activated by annexin-1 and indicate that enhancing the annexin-1–nFPR interaction could be a potent candidate for the treatment of neurological diseases.

2. Materials and methods

2.1. Ethics statement

All animals were handled according to the Council for International Organization of Medical Sciences on Animal Experimentation (World Health Organization, Geneva, Switzerland) and the Huazhong University of Science and Technology guidelines for laboratory animals. The protocol was approved by the Committee on the Ethics of Animal Experiments of Tongji Medical College. All surgery was performed as in the approved protocols, and all efforts were also made to minimize suffering.

2.2. Primary cortical neuronal cultures

Neuronal cultures were prepared from the cortices of embryonic Day 16–18 Sprague–Dawley rats (or embryonic Day 14–16 BALB/c mice) based on previous methods. In brief, dissected cortices were dissociated by a 0.25% trypsin–EDTA (Gibco, Maryland, USA) digestion for 15 min, stopped with 10% fetal bovine serum (FBS, Gibco), and followed by mechanical trituration in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS. Dissociated neurons were plated at a density of 2×10^5 cells/well in neurobasal media supplemented with 2% B-27, 1% penicillin–streptomycin (neuronal culture medium), and 0.5 mM L-glutamine (all from Gibco) on 24-well plates that had been precoated overnight with 50 μg/ml poly-D-lysine (Sigma). Contaminating glial cells were eliminated by treatment with 2 μM cytosine arabinoside (Sigma) from days in vitro (DIV) 2 to 6. The purity of the cultures was verified to be >98% neurons using the neuron-specific marker NeuN. Experiments were performed on DIV7 to DIV10 to ensure morphological and physiological maturity.

2.3. Primary microglial cultures

Mixed glia were prepared from the whole brains of postnatal Day 1–2 Sprague–Dawley rats (or postnatal Day 1–2 BALB/c mice) based on the method of Nakajima et al. (1992). In brief, dissected tissues were dissociated by a 10-min enzymatic digestion with 0.25% trypsin–EDTA, mechanically triturated in DMEM with 10% FBS, and then plated on poly-D-lysine precoated 75 cm^2 tissue culture flasks at a density of 1.0×10^5 to 1.2×10^5 cells/cm^2. After 14–21 DIV, microglial cells were isolated from the mixed glial cultures by mild shaking for 6 h at 150 rpm in a rotary shaker at 37 °C. The floating cells were centrifuged at 1000 ×g at 4 °C for 5 min. The cells were resuspended and plated in DMEM supplemented with 10% FBS on poly-D-lysine-coated plates or coverslips (1×10^5 cells/ml). The purity of adherent cells was verified to be >95% microglia using the microglia-specific marker Iba-1. Microglial cultures were used for experiments 1 DIV after isolation. The DMEM was replaced with neurobasal media (with no B-27 supplements) 24 h prior to the co-culture experiment to match the culture media with that of the primary neurons.

2.4. Chemicals

Unless otherwise mentioned, all chemicals were purchased from Sigma. Peptide Ac2-26 (acetyl-AMVSEFLKQACYIEKQEQEYVQAVK) and the scrambled control (acetyl-YESQFKAVWVE-INTQQMLKFEAEEV) were synthesized based on previously published data by the AngTai Biotechnology Center (Hangzhou, China) using solid-phase stepwise synthesis. Purity was greater than 98% as assessed by high-performance liquid chromatography (HPLC) and capillary electrophoresis (data supplied by the manufacturer). The non-selective nFPR antagonist, N-tert-butoxy-carbonyl-methionyl-leucyl-phenylalanine (Boc-1), and Fpr2 specific antagonist, N-tert-butyloxy-carbonyl-phenyalanine-leucyl-phenyalanine-leucyl-phenyalanine (Boc-2), were obtained from MP Biomedicals (Solon, OH, USA). Boc-1 and Boc-2 were first dissolved in small amounts of DMSO and then diluted in medium (the final concentration of DMSO never exceeded 1% and appropriate controls were included in all experiments).

2.5. Transfection of BV-2 cells with nFPR plasmids and nFPR small interference (si)RNAs

BV-2 cells were plated in six-well plates (Corning Inc., Corning, NY, USA) in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) containing 10% fetal bovine serum (FBS, Gibco) at 3×10^5 cells/well for 24 h before transfection in a humidified atmosphere of 5% CO2 and 95% air at 37 °C. Then, cells were transfected with nFPR-HA (human) cDNA using the expression plasmid, PCDNA3.1$^+$ (Invitrogen) and FUGENE6 (Roche Applied Science) according to the manufacturer's protocol. Transfectants were harvested after 48 h incubation. All these cDNA plasmids were prepared with Endo-free kits (Qiagen, Shanghai, China) to avoid contamination of endotoxins. For nFPR siRNA transfection, prevalidated siRNA duplex sequences selected for use from the three that underwent initial evaluation are as follows: Fpr1, AUAGCC AGCGAAUACAGCAGGUGUC; Fpr2, GGCUCAAACUGAUGAAGAATT; and Fpr3, GAGGGAUCAUCAGGUUCAUTT. Control siRNA used for these experiments contained 48% GC content.

2.6. Oxygen glucose deprivation

The cultures were transferred to an anaerobic incubator with a 5% CO_2 and 95% N_2 atmosphere.The cultures were washed three times with glucose-free extracellular fluid (ECF) and maintained in anoxic condition(5% CO_2, 95% N_2) for 1 h or graded OGD (0 min (control), 30 min, 1 h, 2 h). Following OGD treatment, the cultures were re-oxygenated under normoxic conditions for 24 h before they were collected for assays. In experiments where microglial cultures were exposed to OGD, the same incubation times were applied.

2.7. Measurement of cell viability

For neuronal or neuron-microglia co-cultures, cell viability was assessed by the MTT (thiazolyl blue tetrazolium bromide) assay. Cells were incubated with 0.5 mg/ml MTT for 4 h at 37 °C and 5% CO_2. The cells were solubilized by dimethyl sulfoxide (DMSO) and then read on a plate reader at 560 nm, and the background at 630 nm was subtracted.

2.8. Western blot

Immediately after the treatment, the cultures were washed three times with ice-cold PBS and then lysed in ice-cold RIPA buffer (50 mM Tris, 1 mM EDTA, 1 mM EGTA, 150 mM NaCl) containing 0.8% Triton X-100, 0.2% sodium dodecyl sulfate (SDS), and 1 mM of the protease inhibitor phenylmethanesulfonyl fluoride (PMSF). After centrifugation at 12,000 ×g at 4 °C for 15 min, the lysates were collected. The protein concentration of each lysate was determined with the BCA Protein