Optineurin with amyotrophic lateral sclerosis-related mutations abrogates inhibition of interferon regulatory factor-3 activation.

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**Abbreviations**

ALS, amyotrophic lateral sclerosis

IFN, interferon

IRF3, interferon regulatory factor-3

ISRE, interferon-stimulated response element

MDA5, melanoma differentiation-associated gene 5

TBK-1, TANK-binding kinase-1

TLR3, Toll-like receptor 3

TRAF6, TNF receptor-associated factor 6

TRIF, Toll-IL-1 receptor domain-containing adaptor-inducing interferon-β
Abstract

Optineurin has been shown to be involved in primary open-angle glaucoma. We recently found that optineurin is involved in familial amyotrophic lateral sclerosis (ALS). On the other hand, optineurin has been shown to inhibit transcription factors related to innate immunity such as NF-κB and interferon regulatory factor-3 (IRF3). In the present study, the effect of ALS-associated optineurin mutations on IRF3 activation was investigated. Optineurin inhibited IRF3 activation induced by melanoma differentiation-associated gene 5 or Toll-IL-1 receptor domain-containing adaptor-inducing interferon-β. The inhibition was abrogated by mutations related to ALS but not by a mutation related to glaucoma. Reporter assay indicated that the JAK-STAT signaling pathway was not affected by optineurin. These results show that ALS-related optineurin is involved in the IRF3 activation pathway. Pathogenesis of ALS may be associated with some kind of innate immunity, especially that against virus infection, through IRF3 activation.

Key words: optineurin; ALS; IRF3
Introduction

Amyotrophic lateral sclerosis (ALS) is a progressive neurodegenerative disorder characterized by loss of motor neurons in the primary motor cortex, brainstem and spinal cord [10]. Genes associated with familial ALS have so far been identified include superoxide dismutase 1 [15], angiogenin [5], TAR DNA-binding protein (TDP-43) [17] and FUS/TLS [9, 18]. Recently, we have identified three types of mutations in the gene of optineurin derived from Japanese familial ALS [12].

Optineurin, also called Nemo-related protein NRP, has been reported to be a causative factor of primary open-angle glaucoma [14]. Furthermore, optineurin is a negative regulator of NF-κB activation induced by tumor necrosis factor α [21]. It has also been shown that optineurin forms a complex with TANK-binding kinase-1 (TBK-1) and TNF receptor-associated factor 6 (TRAF6), inhibiting interferon regulatory factor-3 (IRF3) activation and interferon (IFN) production [11].

RNA virus infection is sensed by RIG-I-like receptors such as retinoic acid-inducible gene I (RIG-I) and melanoma differentiation-associated gene 5 (MDA5) or by Toll-like receptors (TLR) such as TLR3, TLR7 and TLR8 (reviewed in [1, 7]), leading to TBK-1 and IRF3 activation. The relationship between ALS and innate immunity especially against virus infection therefore should be
addressed.

In the present study, inhibition of IRF3 activation by optineurin with Q398X(non-sense) and E478G mutations associated with ALS and by optineurin with open-angle glaucoma-related E50K mutation was investigated.

Materials and methods

Cells and plasmids

293T cells (human renal epithelial cells expressing the SV40 large T antigen) were propagated in Dulbecco's modified Eagle's minimal essential medium (DMEM) supplemented with 10% fetal calf serum. An expression plasmid, pcDNA-3xFL-OPTN, possessing 3x FLAG tag and optineurin cDNA under the cytomegalovirus promoter, and its derivatives with mutations in optineurin, Q398X(stop), E478G and E50K, were previously described by Maruyama et al. [17]. p-55C1B-EGFP, which has 8 tandem IRF3-binding motifs upstream of the green fluorescent protein gene, was constructed from p-55C1B [20]. pCAG-FL-MDA5 was constructed by inserting the full-length MDA5 cDNA, derived from pEF.mda5 [2], into the pCAGGS vector under the chicken β-actin promoter [13] with simultaneous addition of a FLAG tag at the N-terminus as described
previously [16]. An expression plasmid for human Toll-IL-1 receptor domain-containing adaptor-inducing interferon-β (TRIF), pEFneo-hTRIF, was described previously [8].

**Reporter assay**

Subconfluent 293T cells in a 35-mm dish were transfected with p-55C1B-EGFP (1 µg) and pcDNA-3xFL-OPTN (0.5 µg) together with pCAG-FL-MDA5 (0.3 µg) or pEFneo-hTRIF (0.1 µg) by using the FuGENE HD reagent (Roche Diagnostics). After 24 h, cell lysates were subsequently prepared and processed for Western blotting as described previously [6, 16] by using an anti-GFP antibody (Santa Cruz Biotechnology sc-8334), anti-FLAG antibody (Sigma-Aldrich M2) or anti-TICAM1 antibody (Abnova PAB9738). Protein bands were detected with peroxidase-conjugated secondary antibody and Immobilon Western chemiluminescent HRP substrate (Millipore). Light intensity of GFP bands was quantitated by an LAS-1000 plus imaging analyzer (Fuji Biomedicals). In some experiments, a reporter plasmid, pISRE-GFP (Qiagen), was used and IFN-α (Mochida Pharmaceuticals) was added to the culture medium at 6 h post-transfection.
Results

IRF3 reporter activation, which was induced by overexpression of MDA5, was inhibited by wild-type optineurin (Fig. 1A). However, optineurin with the ALS-related Q398X(stop) or E478G mutation did not inhibit IRF3 activation. On the other hand, the glaucoma-related E50K optineurin inhibited IRF3 activation, as did wild-type optineurin (Fig. 1A). When signal transduction was induced by overexpression of TRIF, an adaptor of TLR3 [19], similar results were obtained (Fig. 1B).

Interferon-stimulated response element (ISRE) reporter activation, which is further downstream of IRF3 in IFN response (Fig. 2), was also inhibited by wild-type and E50K optineurin when the signal was induced by overexpression of MDA5 (Fig. 1C). Q398X and E478G optineurin, however, did not inhibit activation of the ISRE reporter. This effect of optineurin on ISRE activation did not occur downstream of the IFN-α/β receptor, since a difference was not observed by the addition of IFN-α to the medium (Fig. 1D, 2). These results indicated that optineurin inhibited IFN signal transduction upstream of IRF3 and that mutations related to ALS abrogated the inhibitory capacity.
**Discussion**

The optineurin mutant Q398X(stop) lacks the C terminus, causing a deletion of the coiled-coil region that is essential for binding with ubiquitin and some host factors [12]. The E478G mutant lacks binding capacity with ubiquitin. These familial ALS-related mutants have been shown to abrogate inhibition of NF-κB activation by optineurin [12]. The present study revealed that these mutants also abrogated inhibition of IRF3 activation. On the other hand, such an effect of the open-angle glaucoma-related E50K mutant was not observed. It is presumed that the binding activity of optineurin with ubiquitin is primarily required for suppression of IRF3 activation, and the coiled-coil region for interaction with other molecules may also be required.

Optineurin affected the signal transduction pathway to activate IRF3 but not the JAK-STAT pathway downstream of the IFNα/β receptor. This is consistent with the finding that optineurin can form a complex with TBK-1 and TRAF6 [11], demonstrating the point of inhibition by optineurin in innate immunity.

These effects were observed when the signal was induced by a RIG-I-like intracellular RNA sensor, MDA5, and by expression of TRIF, an adaptor of an extracellular RNA sensor, TLR3. Both mimic a part of the mechanism by which IRF3 activation and IFN-β production are induced.
after RNA virus infection [1]. In the presence of ALS-related optineurin mutants, some kind of RNA virus infection may activate IRF3 excessively, leading to strong induction of IFN-β expression.

Simultaneously, excess IRF3 activation is thought to lead to apoptosis of cells. It is known that IRF3 activation leads to apoptosis probably through Bax transcription factor [3]. If this occurs in neurons, it may indicate a close relationship between unknown viral infection and neuronal cell death in familial ALS. Recently, Douville et al. [4] reported transcriptional activation of a human endogenous retrovirus (HERV-K) in ALS patients. The viral reverse transcriptase mRNA may further suggest synthesis of other RNA species that can induce IRF3 and NF-κB activation as a pathogen-associated molecular pattern. Alternatively, RNA viruses that cause acute infection may play a role in induction of the IRF3 pathway in motor neurons.

In summary, optineurin inhibited IRF3 activation as well as NF-κB activation, and the inhibition was abrogated by optineurin mutants derived from familial ALS. This may indicate that innate immunity, especially that against viruses, is related to ALS pathogenesis. This remains to be elucidated in the future.
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Legends of figures

Fig. 1. (A) IRF3 reporter assay. 293T cells were transfected with p-55C1B-EGFP, pCAG-FL-MDA5, and pcDNA-3xFL-OPTN WT, Q398X(stop), E478G or E50K. After 24 h, cell lysates were prepared and processed for Western blotting by using an anti-GFP antibody as well as an anti-FLAG antibody. Data from three independent experiments were plotted on a graph. Error bar represents standard deviation. (B) Experiments similar to A except that pEFneo-hTRIF was transfected instead of pCAG-FL-MDA5. (C) ISRE reporter assay. 293T cells were transfected with pISRE-EGFP, pCAG-FL-MDA5, and an optineurin-expressing plasmid. After 24 h, cell lysates were prepared and processed for Western blotting as described in A. (D) 293T cells were transfected with pISRE-EGFP and an optineurin-expressing plasmid. After 6 h, IFN-α was added to the culture medium, and after a further 18 h, cell lysates were prepared and processed for Western blotting.

Fig. 2. Schematic view of interferon responses against RNA viruses. CBP: CREB-binding protein. Tyk2: tyrosine kinase 2, IRF9: interferon regulatory factor-9. The other abbreviations are mentioned in the text.
Virus

TLR3

TRIF

MDA5

RIG-I

IRF3

IKKε

Tyk2

Jak1

STAT1

STAT2

IRF9

CBP/p300

Optineurin

IRF3

IFN-β promoter

ISRE

cytosol

nucleus