Apoptosis signal-regulating kinase 1 regulates the expression of caspase-11

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Abstract

Caspase-11 is an inducible caspase involved in the regulation of cell death and inflammation. In the present study, we examined whether apoptosis signal-regulating kinase 1 (Ask1)-mediated signaling pathway is involved in the expression of caspase-11 induced by lipopolysaccharide (LPS). We found that the induction of caspase-11 was suppressed by the inhibitors of NADPH oxidase (Nox) or knockdown of Nox4 that acts downstream of toll-like receptor 4 and generates Ask1-activating reactive oxygen species. Overexpression of dominant negative tumor necrosis factor receptor associate factor 6 also suppressed the induction of caspase-11. Importantly, knockdown or dominant negative form of Ask1 suppressed the induction of caspase-11 following LPS stimulation. Taken together, our results show that Ask1 regulates the expression of caspase-11 following LPS stimulation.

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1. Introduction

Caspase-11 is a murine caspase whose expression is regulated mainly at a transcriptional level [1]. It is induced by various cellular insults and under pathological conditions such as lipopolysaccharide (LPS)-induced septic shock, brain ischemia, experimental autoimmune encephalomyelitis, and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-induced Parkinson’s disease models in mice [1–4]. Following induction, it activates caspase-1 by direct interaction and caspase-3 by proteolytic cleavage. Thus caspase-11 can regulate both inflammatory response via cytokine maturation and apoptosis via caspase-3 activation depending on the cellular context [2,5]. Since caspase-11 is involved in many disease conditions with pathological apoptosis and inflammation, understanding of its induction mechanism is necessary to develop therapeutics targeting caspase-11 for the treatment of the related diseases. It was reported that proinflammatory mediators like LPS and interferon γ (IFNγ) induce the expression of caspase-11 by activating nuclear factor (NF)-κB and signal transducer and activator of transcription 1 (STAT1), respectively [6]. A following study showed that LPS-induced activation of NF-κB leading to caspase-11 expression is mediated by a signaling cascade involving Toll-like receptor 4 (TLR4)/myeloid differentiation primary response gene 88 (MyD88) [7]. It was also reported that the activation of p38 mitogen-activated protein kinase (MAPK) in C6 rat glial cells, c-Jun N-terminal kinase (JNK) in mouse embryonic fibroblasts (MEFs) and C/EBP homologous protein in mice was required for the induction of caspase-11 [8–10]. Because of its inducibility, caspase-11 can be a good target of therapeutics against many diseases accompanying excessive apoptosis and inflammation.

Apoptosis signal-regulating kinase 1 (Ask1) is a mitogen-activated protein kinase kinase kinase (MAPKKK) activated by various cell damaging signals including LPS, reactive oxygen species (ROS), endoplasmic reticulum stress, influx of calcium ions, and molecules like tumor necrosis factor α (TNFα) and Fas ligand [11]. Following activation, Ask1 regulates various cellular events like cell death, differentiation, and TLR4-mediated innate immunity [11,12]. In unstimulated cells, Ask1 activation is suppressed by thioredoxin (Trx) but following oxidative stress or engagement of TLR4 or TNF receptor 1, Trx dissociates from the Ask1/TNF receptor associated factor 2/6 (TRAF2/6) complex and activates Ask1 [11]. For this activation, ROS has been reported to be a common second messenger [12]. After LPS stimulation, it was suggested that ROS is generated by NADPH oxidase 4 (Nox4)-containing Nox complex that directly interacts with TLR4 [13]. Ultimately, the activated Ask1 activates p38 MAPK and JNK [11].

Since caspase-11 and Ask1 are activated by similar stimuli, share common signaling molecules, and exhibit similar functions in stress and immune response, we examined whether Ask1 plays a role in the induction of caspase-11. We report here the evidence...
that Ask1 regulates the induction of caspase-11 following LPS stimulation.

2. Materials and methods

2.1. Reagents and antibodies

Anti-caspase-11 antibodies were from Abcam (Cambridge, UK), anti-Ask1 antibodies from Cell signaling Technology (Danvers, MA). Secondary antibodies were from Jackson ImmunoResearch Laboratories (West Grove, PA). Cytokines were purchased from Millipore (Billerica, MA). Cell culture media, fetal bovine serum (FBS) and other culture supplements were from JBI (Daegu, Korea). All other reagents were from Sigma–Aldrich unless stated otherwise (St. Louis, MO).

2.2. Cell culture and transfection

MEFs were cultured as described previously [9]. Transfection was carried out using TransIT-LT reagent according to the manufacturer’s protocol (Mirus Bio, Madison, WI). To isolate peritoneal macrophages, mice were peritoneally injected with thioglycollate medium (4%, 1 ml/mouse). After 4–6 days, mice were sacrificed and peritoneal exudate cells were collected using an 18-gauge needle and 1% FBS/RPMI 1640 medium. Cells were plated and incubated for 3 h and unattached cells were washed away. Astrocytes were cultured from ICR mouse pups at postnatal day 1 as described previously [14] with minor modifications.

2.3. Immunoblots

Proteins (25–30 μg) prepared from MEFs were subjected to 10% SDS–polyacrylamide gel electrophoresis and immunoblot assay following standard protocols.

2.4. Gene knockdown by oligonucleotide siRNA

Small interfering RNA (siRNA) duplexes for Nox4 (siRNA 1392781) and Ask1 (siRNA 100233), and negative controls were purchased from Bioneer (Daejeon, Korea, sequences available upon request). For siRNA transfection, 100 pmoles of oligonucleotide siRNA duplexes were mixed with 5 μl Lipofectamine 2000 (Invitrogen, Carlsbad, CA) in 200 μl TOM and added onto MEFs (5 × 10^5/ml) grown on 35 mm dish. After 4 h incubation, the medium was replaced with normal growth medium.

2.5. Dual luciferase assay

MEFs (5 × 10^5/ml) grown on 24-well culture plates were transfected with caspase-11 promoter-luciferase plus pRL-TK expression vectors (5:1) using TransIT-LT1 transfection reagents. Following stimulation or co-transfection with other plasmid DNAs such as Ask1 expression vector, the cells were lysed and sequentially measured for the firefly and Renilla luminescence using Promega Dual-Luciferase Reporter Assay kit (Madison, WI) according to the manufacturer’s manual.

2.6. RT-PCR

Total RNA from tissues and cultured cells was prepared using RNeasy minikit (Qiagen, Valencia, CA). cDNA was synthesized using the Moloney Murine Leukemia Virus Reverse Transcriptase (Promega, Madison, WI) according to the manufacturer’s protocol. For the detection of caspase-11 mRNA, forward (5’-GAAGCCATTTGGAGACTCA-3’) and reverse (5’-GCTTACCCAGGAGCAGC-3’) primer pairs were used.

3. Results

To examine a possible role of Ask1 in the induction of caspase-11 following LPS stimulation, we first confirmed the involvement of TLR4 which is an LPS receptor and known to convey signal to activate Ask1 [11]. Jung et al. [7] have shown that caspase-11 mRNA is not detected in the microglia of C3H/Hej mice that have spontaneous mutation in TLR4 gene. Consistent with this earlier observation, caspase-11 induction was not detected in the MEFs of C3H/Hej mice at a protein level following LPS stimulation (Fig. 1A). However, caspase-11 induction by interleukin 1β (IL-1β), TNFα, or IFNγ was normal in the MEFs of C3H/Hej mice (Fig. 1A). The requirement of TLR4 in the LPS-induced expression of caspase-11 was also confirmed in the astrocytes and peritoneal macrophages cultured from C3H/Hej mice (Fig. 1B). These results show that TLR4 mediates the LPS signal to induce caspase-11 in MEFs.

It has been reported that Ask1 is activated by ROS and the stimulation of TLR4 can generate ROS by Nox complex interacting with the TLR4 [12,13]. To determine whether Nox-mediated generation of ROS is required for the induction of caspase-11, we first examined whether a general antioxidant like N-acetylcysteine (NAC) can attenuate the LPS-induced expression of caspase-11. The MEFs pretreated with NAC exhibited reduced level of caspase-11 protein following LPS stimulation compared to the MEFs stimulated with LPS alone (Fig. 2A). This suggests that the ROS generated by LPS stimulation play a role in the signaling for the caspase-11 induction. To identify a possible source of these ROS, the MEFs were pretreated with inhibitors of Nox, i.e., diphenyleneiodonium (DPI) and apocynin and then stimulated with LPS, and the expression level of caspase-11 was examined. As shown in Fig. 2B, both DPI and apocynin efficiently suppressed the LPS-induced expression of caspase-11, indicating activation of Nox and the production of ROS can mediate the induction signaling for caspase-11. Since there has been arguments regarding the specificity of apocynin and DPI as Nox inhibitors [15], we examined the knockdown effect of
Nox4 that has been shown to interact directly with TLR4 [13] to further confirm the involvement of Nox in the caspase-11 induction signaling. As shown in Fig. 2C and D, the knockdown of Nox4 attenuated the induction of caspase-11 at both mRNA and protein levels after LPS stimulation. These results suggest that the activation of Nox4 following TLR4 engagement by LPS mediates a signal to induce caspase-11.

It has been shown that TRAF6 activates Ask1 by forming a complex following TLR4 activation and the formation of this TRAF6–Ask1 complex is ROS-dependent [12]. Although there has been no direct evidence showing activation of Nox4 is the source ROS required for the TRAF6–Ask1 complex, involvement of Nox4 in TLR4 signaling for the caspase-11 induction prompted us to examine whether TRAF6 plays a role in the regulation of caspase-11 induction following Nox activation. To examine whether TRAF6 is involved in the induction of caspase-11, wild-type or N-terminal deletion (amino acids 1–288) dominant negative TRAF6 [16] was overexpressed in the MEFs and the level of caspase-11 protein was compared following LPS stimulation. As shown in Fig. 3A, overexpression of the dominant negative TRAF6 attenuated the expression of caspase-11, indicating TRAF6 plays a role to induce caspase-11. The activation of TRAF6 in the caspase-11 induction signaling seemed to be downstream of ROS generation since the suppression of caspase-11 induction by NAC was reversed by overexpression of TRAF6 (Fig. 3B).

It has been reported that ROS generated following TLR4 activation triggers the formation of TRAF6–Ask1 complex and this results in the activation of Ask1 [12]. Since we observed the signaling molecules upstream of Ask1 were involved in the regulation of caspase-11 induction, we then examined whether Ask1 plays a role to induce caspase-11. First, we compared the effect of overexpression of wild-type vs. dominant negative form of Ask1 (K709M) on the promoter activity of caspase-11. As shown in Fig. 4A, overexpression of the wild-type Ask1 activated the caspase-11 promoter while that of the dominant negative Ask1 did not. Furthermore, knockdown of Ask1 suppressed the LPS-induced upregulation of caspase-11 (Fig. 4B), confirming the role of Ask1 in the induction of caspase-11. Consistently with other reports [11,12], the activation of Ask1 appeared to be downstream of TRAF6 activation since the suppression of caspase-11 induction by dominant negative TRAF6 was reversed by overexpression of Ask1 (Fig. 4C). These results indicate that Ask1 mediates the caspase-11 induction signaling after LPS stimulation.

4. Discussion

In the present study we showed evidence indicating that Ask1 mediates the LPS-induced signal to upregulate caspase-11 expression in MEFs. Following LPS stimulation, we observed evidence of involvement of TLR4–Nox4–ROS–TRAF6–Ask1 signaling cascade in the induction of caspase-11. Previously, a signaling cascade for the caspase-11 induction has been studied only partially from the LPS receptor to NF-kB in several studies. In the upstream of the caspase-11 induction signaling, a requirement of TLR4 in the LPS-induced caspase-11 expression in microglia has been reported [7]. In this report, a signaling via MyD88-IRAK-TRAF6 has been implicated to mediate the TLR4 signal for the induction of caspase-11 but this was not directly tested in the study [7]. Among the downstream MAPKs, p38 MAPK and JNK have been shown to...
mediate the caspase-11 induction [8,9]. The involvement of p38 was further supported by the observation that an inhibitor of p38 MAPK attenuated the cell death and induction of caspase-11 in the rat substantia nigra following LPS injection [17]. Ask1 has been also known to activate p38 MAPK [12]. Therefore, it is highly probable that caspase-11 is induced by LPS via TLR4–Nox4–ROS–p38 MAPK. However, Ask1-induced expression of caspase-11 cannot be ruled out.

MAPKKKs downstream of TLR4–TRAF6 other than Ask1 can be transforming growth factor-beta-activated kinase 1 (TAK1) or MAPK/extracellular signal-regulated kinase kinase kinase 3 (MEKK3) [11]. Since caspase-11 promoter has been known to be activated by NF-κB that is a direct target of TAK1 [6,14], one can speculate that TAK1 is activated downstream of TLR4–TRAF6 and activates NF-κB via IKK activation [18]. However, Mochida et al. [19] reported that Ask1 inhibits IL-1-induced NF-κB activation by disrupting TRAF6–TAK1 interaction. If this is also true in case of LPS-induced activation of Ask1, a signal divergence for TAK1 activity may not be possible. However, both NF-κB and Ask1 do activate following LPS stimulation [14,20], the possibility that Ask1 suppresses NF-κB activation by disrupting TRAF6–TAK1 interaction is very low in LPS-signaling. Thus, involvement of MAPKKKs other than Ask1 in the LPS-induced caspase-11 expression remains to be studied.

Transcription factors for the caspase-11 have been reported to be NF-κB or STAT1 following stimulation with LPS or IFNγ, respectively [13]. It has been shown that LPS-induced activation of Ask1 leads to the activation of p38 MAPK but not JNK [18] and p38 MAPK does not directly activate NF-κB although a pharmacological inhibition of p38 MAPK attenuates the NF-κB activation [20]. Therefore, Ask1-induced expression of caspase-11 may be mediated by transcription factor(s) other than NF-κB. It has been reported that p38 MAPK regulates the expression of pro-inflammatory cytokines by activating AP-1 [20]. Indeed, the caspase-11 promoter [6] has putative AP-1 binding sequences when scanned by several on-line promoter analysis programs such as TFSEARCH or TESS. Therefore, it also remains to be determined which transcription factor mediates the LPS–Ask1 signal to induce caspase-11 gene.

Since caspase-11 can activate both inflammatory response by pro-inflammatory cytokines and apoptosis under pathological conditions [2,5], a detailed understanding of the mechanism of its induction and activation can be a prerequisite for the development of therapeutic strategy to inhibit pathological functions of caspase-11 without interfering with its possible physiological functions.

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References


