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ABSTRACT

The NLRP3 inflammasome is activated by a variety of external or host-derived stimuli and its activation initiates inflammatory response through caspase-1 activation resulting in inflammatory cytokine IL-1β maturation and secretion. The NLRP3 inflammasome activation is a kind of innate immune response most likely mediated by myeloid cells acting as a host defense mechanism. However, if the activation is not properly regulated, excessive inflammation induced by overactivated NLRP3 inflammasome can be detrimental to the host, causing tissue damage and organ dysfunction, eventually causing several diseases. Previous studies suggested that mitochondrial damage may be a cause of NLRP3 inflammasome activation and autophagy which is a conserved self-degradation process negatively regulates the NLRP3 inflammasome activation. Recently, mitochondria-selective autophagy termed mitophagy has emerged as a central player for maintaining mitochondrial homeostasis by elimination of damaged mitochondria, leading to the prevention of hyperinflammation triggered by NLRP3 inflammasome activation. In this review, we will first focus on the molecular mechanisms of NLRP3 inflammasome activation and the NLRP3 inflammasome-related diseases. And then we will discuss about autophagy especially mitophagy as a negative regulator of the NLPP3 inflammasome activation by examining recent advances in research.

INTRODUCTION

The immune system is a host protection mechanism against invading pathogens such as viruses and bacteria. Macrophages, the front line defenders who recognize invading pathogens, not only kill the pathogens by phagocytosis but also initiate inflammation producing inflammatory cytokines upon infections. The maturation and secretion of IL-1β, one of the strongest pro-inflammatory cytokine, are enhanced by inflammasome in macrophages leading to inflammation and cell death (1, 2).

Inflammasomes are molecular platforms that trigger activation of caspase-1 leading to pro-inflammatory cytokine maturation. One of the best characterized inflammasome is NLRP3 inflammasome. Even though NLRP3 inflammasome activation is important for protecting cells from pathogenic microbes, excessive NLRP3 inflammasome may cause hyperinflammation resulting in tissue damage and organ failure. NLRP3 inflammasome is also activated by danger signals released from stressed cells and dysregulation of NLRP3 inflammasome activation causes many diseases such as neurodegenerative diseases, metabolic diseases and sepsis. Therefore, mechanisms to control NLRP3 inflammasome activation are necessary for health.

Upon bacterial infection, innate immune cells such as macrophages and neutrophils take up bacteria and generate reactive oxygen species (ROS) (3-5). Two main sources of ROS is NADPH oxidase-mediated ROS and mitochondria-derived ROS in innate immune cells (6-8). Some studies proposed that mitochondrial dysfunction is closely associated with NLRP3 inflammasome activation. Infection causes mitochondrial damage through an unknown mechanism and the damaged mitochondria release mitochondrial DNA (mtDNA) and mitochondrial reactive ROS (mtROS) and they are thought to work as danger signals(9, 10). Cells have a regulatory mechanism ‘mitophagy’, a mitochondria-selective autophagic process

to eliminate damaged or unwanted mitochondria, to maintain mitochondrial homeostasis against stress. This review will discuss how the balance between pro-inflammatory response and anti-inflammatory response is maintained in cells focusing on NLRP3 inflammasome activation and mitophagy.

INFLAMMASOME

Inflammation is one of the first innate immune responses to combat foreign organisms entering the body that are able to cause diseases (11). Most foreign organisms express various pathogen-associated molecular patterns (PAMPs) in their cell wall or on their cell surface (12). Therefore it is possible that a host can distinguish from self to non-self by recognizing PAMPs. Moreover, under stressed conditions, cells release danger signals called danger-associated molecular patterns (DAMPs) to notify their urgent situation to the immune system. PAMPs and DAMPs are recognized by innate immune receptors pattern-recognition receptors (PRRs), such as Toll-like receptors (TLRs) and nucleotide-binding and oligomerization domain (NOD)-like receptors (NLRs) (13). Activation of PRRs leads to Nf-κB activation and inflammatory cytokine production. A lot of studies showed that inflammasome is a key regulator of inflammation (1, 14-17). Most inflammasomes contain NLR proteins and the NLR family can be classified into 3 subtypes based on their domain structures: the NODs (NOD1-5 and CIITA), the NLRPs (NLRP1-14) and the IPAF (IPAF also known as NLRC4, and NAIP) subtypes (18). NLR family commonly contains a central nucleotide-binding and oligomerization (NACHT) domain. Most of NLRs also contain C-terminal leucine-rich repeats (LRRs) and N-terminal caspase recruitment domains (CARD) or pyrin domains (PYD). NACHT plays an essential role in activation and formation of the signaling complex and LRR mediates ligand sensing, while CARD and PYD function in homotypic protein-protein interaction for downstream events (18, 19). A non-NLR inflammasome member,

absent in melanoma-2 (AIM2), was later identified that can form an inflammasome composed of AIM2, ASC and caspase-1. The functions and mechanisms of many NLR family members are poorly understood but some of them are well-characterized. For example, NOD1 and NOD2 are receptors for bacterial peptidoglycan fragments. NOD1 and NOD2 recognize D-glutamyl-meso-diaminopimelic acid (DAP) and muramyl dipeptide (MDP), respectively. Upon sensing them, NOD1 and NOD2 oligomerize and recruit RIP2 via CARD-CARD interactions triggering inflammatory response (20, 21). CIITA uniquely acts as a transcription factor playing a key role in the regulation of class II MHC genes (22). IPAF inflammasome is activated in response to gram-negative bacteria containing type III or IV secretion systems, such as *Psudomonas aeruginosa, Salmonella typhimurium* and *Shigella flexneri* (23-25). AIM2 inflammasome is activated by sensing cytosolic double-stranded DNA (dsDNA) through the HIN-200 domain of AIM2(26, 27). As shown here, each inflammasome is activated by different stimuli but more studies about other NLR family members are needed. In this review, the NLRP3 inflammasome which is the most characterized among inflammasome members will be discussed in detail in following sections.

NLRP3 INFLAMMASOME

NLRP3 inflammasome is activated in response to abundant stimuli and 2 distinct steps are required for this activation. First, Nuclear factor-κB (Nf-κB) activation by TLR ligands increases NLRP3 and pro-1L-1β. This step is called ‘priming’. Second, activating stimuli derived from microbes or host induce the assembly of NLRP3 inflammasome components (17). NLRP3 inflammasome is composed of NLRP3, apoptosis-associated speck-like protein containing a CARD (ASC) and pro-caspase-1. ASC is an inflammasome adaptor containing N-terminal PYD and C-terminal CARD. NLRP3 inflammasome is assembled following these steps: PYD of NLRP3 is oligomerized with ASC through PYD-PYD interaction.

Subsequently, pro-caspase-1 is recruited and interact with ASC through CARD-CARD interaction (28). After formation of NLRP3 inflammasome, pro-caspase-1 is autocleaved to active caspase-1 and the active caspase-1 maturates cytokine such as IL-1β to its bioactive and secreted form (17). Currently, various sterile DAMP signals and pathogens including mitochondrial ROS, mitochondrial DNA, potassium efflux, MDP, monosodium urate (MSU), cholesterol crystals, cathepsins, influenza virus, *Salmonella typhimurium*, *Mycobacterium tuberculosis* and others are known to activate NLRP3 inflammasome (29). Compared to NLRP3 inflammasome activators, negative regulators of NLRP3 inflammasome are relatively less discovered. Recent studies suggest that nitric oxide (NO), Ca^{2+} and cyclic AMP negatively regulate NLRP3 inflammasome but the detailed mechanisms are unclear (30, 31). If the upregulated NLRP3 inflammasome activation is not downregulated, inflammation will be persistently induced by NLRP3 inflammasome and contribute to diverse diseases

NLRP3 INFLAMMASOME IN DISEASE; NEURODEGENERATIVE DISEASE

Dysregulation of NLRP3 inflammasome activation is observed in neurodegenerative diseases. Alzheimer's disease (AD), a representative neurodegenerative disease, is characterized with amyloid-β plaque accumulation. Halle *et al*. identified using *in vitro* and *in vivo* mouse model that amyloid-β causes lysosomal damage releasing cathepsin B and the cathepsin B activated NLRP3 inflammasome (32). In addition to the mouse study, recently performed human study showed that increased active caspase-1 expression is observed in the brains of AD patients (33). It has been reported that not only AD but also Parkinson's disease (PD) are associated with NLRP3 inflammasome. Accumulation of Lewy bodies (LB) formed by α-synuclein (αSyn) aggregation is a main pathogenesis of PD. A recent study revealed that α-synuclein induces synthesis of pro-IL-1β by interaction with TLR2 and activates NLRP3 inflammasome resulting in caspase-1 activation and IL-1β maturation in human primary

monocytes (34). On the other hand, it is reported that mitochondrial dysfunction may lead to neurodegenerative diseases (35). These studies indicate that both NLRP3 inflammasome and mitochondrial dysfunction are involved in neurodegenerative diseases though more studies are required to clarify the relationship among NLRP3 inflammasome, mitochondria and neurodegenerative diseases.

NLRP3 INFLAMMASOME IN DISEASE; METABOLIC DISORDER

It has been known that High fat diet (HFD) can induce Type 2 diabetes (T2D) and obesity and chronic inflammation is thought to contribute to (T2D) and obesity (36). So there is a possibility that NLRP3 inflammasome which can lead to chronic inflammation is related to these metabolic diseases. Recently Lee *et al*. observed activated NLRP3 inflammasome in T2D patients and found that mitochondrial ROS is involved in this phenomenon(37). Other groups showed that saturated fatty acid-induced inflammation causes mitochondrial impairment in adipocytes while saturated fatty acid can upregulate NLRP3 inflammasome activation (38, 39). Also, higher level of NLRP3 inflammasome components were detected in obese patients, and active caspase-1 were increased with obesity development in adipose tissues (40). Interestingly, caspase-1 deficient mice gained less weight and they had less adipose tissue formation in HFD-induced obesity mouse model (41). These data strongly suggest that NLRP3 inflammasome and mitochondrial dysfunction are closely linked to metabolic disorders.

NLRP3 INFLAMMASOME IN DISEASE NLRP3; SEPSIS

Sepsis is a life-threatening systemic inflammatory condition caused by a host immune response to microbial infection. Normally, increased inflammatory response to infection should be resolved in a timely manner but when it gets out of control, exaggerated activation

of inflammasome produces excessive inflammatory cytokines leading to sepsis. A few studies provided evidence that mitochondrial dysfunction is associated with sepsis. Damaged mitochondria of the cells treated with NLRP3 inflammasome activators release danger signals such as mtROS and mtDNA and these signals can activate NLRP3 inflammasome (9, 10). Supporting these, increased mtDNA and inflammatory cytokines were detected in septic plasma samples (42). This evidence shows that both NLRP3 inflammasome and mitochondria contribute to sepsis.

MITOCHONDRIA AND NLRP3 INFLAMMASOME

Mitochondria are double membrane organelles that play pivotal roles in cells including energy production and regulation of cell death. In addition, recent studies revealed that mitochondria are also involved in innate immune responsev(43, 44). Mitochondria are main sites of reactive oxygen species (ROS) production. Mitochondrial ROS are not just byproducts of aerobic respiration but also participate in signaling pathway. However, overproduced ROS cause cell damage and contribute to pathologies. Some studies showed that ATP and monosodium urate (MSU) crystal increase ROS production and these activate inflammasome (45, 46). Silica and asbestos also increase of ROS production and activate NLRP3 inflammasome (47). In another study, increased mtROS by inhibition of mitochondrial complex I or complex III, triggered NLRP3 inflammasome activation indicating mtROS can be a main cause of NLRP3 inflammasome activation (48, 49). Not only mtROS, but mtDNA also activates NLRP3 inflammasome. Under normal conditions, mtDNA is located in mitochondria. However, when mitochondria is damaged, mtDNA is released into the cytoplasm. Shimada *et al*. found oxidized mtDNA released from damaged mitochondria directly induces NLRP3 inflammasome (10). Mitochondrial outer membrane proteins are also thought to activate NLRP3 inflammasome. Mitofusins, mitochondrial outer

membrane proteins which are required to mitochondrial fusion, activate NLRP3 inflammasome in influenza and encephalomyocarditis virus (EMCV) infection while another mitochondrial outer membrane protein MAVS recruits NLRP3 to mitochondria and activates inflammasome activation in response to ATP, nigericin and poly I:C(50). All of these data indicate that mitochondria play a crucial role in NLRP3 inflammasome activation.

AUTOPHAGY AND NLRP3 INFLAMMASOME

As described above, there is a lot of evidence that damaged mitochondria contribute to disease including NLRP3 inflammasome-related diseases. Therefore, the importance of mitophagy which is a process to remove damaged mitochondria becamse considered a key process to regulate NLRP3 inflammasome activation. Despite the importance of mitophagy, it has mostly just been studied in mitochondria-related gene overexpressed mammalian cell lines in the past few years. To investigate its mechanism and function accurately, more *in vitro* and *in vivo* studies are required. Knowledge about macroautophagy (hereafter autophagy) is prerequisite to understanding mitophagy. Autophagy is a self-degradation process to recycle cellular components under stressed conditions such as lack of nutrition. Autophagy has been regarded as a nonselective process in the past, however, accumulating evidence show it can be selective to remove specific proteins and damaged organelles such as mitochondria under certain conditions(51-53). Autophagy consists of four sequential steps: initiation of autophagosome formation, elongation and closure of autophagic membrane, fusion between autophagosome and lysome, and degradation. In many cellular cases, autophagic induction is regulated by the *mammalian* target of rapamycin (mTOR) and the AMP-activated protein kinase (AMPK). mTOR inactivates unc-51-like kinase 1/2 (ULK1/2) by phosphorylation under basal conditions, but mTOR is inhibited under stressed condition letting ULK1/2 be modified to their active forms by AMPK phosphorylation initiating

autophagy. Next, VPS34 lipid kinase complex and phosphatidylinositol 3-phosphate (PI(3)P) are recruited to complete autophagosome formation. In the step of elongation and closure of autophagic membrane, the ubiquitin-like protein ATG12 and ATG5 are conjugated and then the conjugate forms E3-like ligase complex with ATG16L1. After that, ATG8 (LC3 and GABARAP subfamilies) is conjugated to the lipid phosphatidylethanolamine by the ATG16L1 ligase complex and in the case of LC3, LC3-I form changes to LC3-II form which is often used as a autophagosome marker (44, 54). VPS34–Beclin 1 complex containing ultraviolet radiation resistance-associated gene protein (UVRAG) is involved in the step of fusion between autophagosome and lysosome. Once autophagosome and lysosome are fused to autolysosome, the cargo in the autolysosome is degraded through the lysosomal hydrolase activity (55). When there are certain proteins or organelles to be eliminated, they are ubiquitinated by E3 ubiquitin ligases and the ubiquitin chains serve as ‘eat-me’ signals. Then adaptor proteins such as p62 and NBR1, which contain ubiquitin-associated UBA domain and LC3-interacting region (LIR), capture the ubiquitinated cargos and recruit LC3-II to proceed selective autophagy(54). Accumulating evidence has shown that autophagy is involved in innate immune response and NLRP3 inflammasome activation. Inhibition of autophagy by 3MA which is a PI3K inhibitor increased mtROS production resulting in NLRP3-dependent IL-1β secretion in the absence of inflammasome stimul i(56, 57). Similarly, treatment of 3MA blocking autophagosome formation in *Mycobacterium tuberculosis*-infected cells led to more IL-1β secretion (58). Consistent with these data, Bone marrow-derived macrophages from LC3 knockout mice and Beclin 1 knockout mice had more damaged mitochondria producung mtROS, and had more caspase-1 activation leading to more IL-1β secretion upon NLRP3 inflammasome activation (9). Also, Atg16L1 defienct mice were more susceptible to dextran sulfate sodium-induced colitis as a result of enhanced inflammasome activation (58). These data show that autophagy negatively regulates inflammasome activation. Moreover, as

damaged mitochondria are more detected under autophagy inhibition (9, 56, 57), it is plausible to assume that elimination of damaged mitochondria by autophagy is important to prevent excessive NLRP3 inflammasome activation.

MITOPHAGY AND NLRP3 INFLAMMASOME

As mentioned above, Mitochondrial damage causes NLRP3 inflammasome, and mitophagy can remove damaged mitochondria selectively (Figure 1). Since mitochondria are evolved from ancient bacteria, comparison of differences between mitophagy and intracellular bacteria-selective autophagy referred as to xenophagy can provide valuable informations to better understand mitophagy. Distinguishing between self and non-self is achieved by different ‘eat-me’ signals and cargo receptors (52, 59). To date, at least four cargo receptors have been studied in detail; NDP52, p62, NBR1 and Optineurin (59). Invading bacteria can damage their host vacuoles in which the bacteria replicate exposing glycans from the host vacuoles, and the glycans are recognized by a danger receptor Galectin-8. Galectin-8 functions as an ‘eat-me’ signal and interacts with NDP52 inducing xenophagy by recruiting LC3 attached to phagophores (60). NDP52 can also directly bind to ubiquitinated bacteria and recruit the autophagic machinery. Similarly, p62, NBR1 and Optineurin serve to connect ubiquitinated bacteria with LC3 faciliating autophagy. How the engulfed bacteria are ubiquitinated is still largely unknown but LRSAM1 has been identified as an E3 ubiquitin ligase that ubiquitinates *Salmonella typhimurium* recruiting cargo receptors (61). Another E3 ubiquitin ligase PARKIN has also been reported to be involved in xenophagy. PARKIN deficient mice and flies were susceptible to intracellular bacterial infections inducing less autophagy, and these indicates that PARKIN plays a crucial role in xenophagy (62). Interestingly, PARKIN is the best known E3 ubiquitin ligase in mitophagy. The PINK-PARKIN pathway is important in mitophagic processs (63, 64). PINK1 is a serine/threonine

kinase containing mitochondrial targeting sequence in N-terminus. Normally, PINK1 is imported into mitochondria and anchored at the inner mitochondrial membrane (IMM) and then degraded by mitochondrial proteases. However, when mitochondria is damaged, PINK1 can't be imported to IMM but instead it is accumulated on outer mitochondria membrane (OMM) (65). The accumulated PINK1 recruits a cytosolic E3 ubiquitin ligase PARKIN and activates PARKIN by phosphorylation. Activated PARKIN ubiquitinates certain mitochondrial proteins or PARKIN itself. Then, the mitochondrial ubiquitination acts as an 'eat me' signal and can be recognized by adaptor p62 through its ubiquitin binding domain. Since p62 also has LC3 interacting motif, p62 binds with LC3 on autophagosome and facilitate degradation of damaged mitochondria (44). However, not all mitophagy is processed by PINK-PARKIN pathway. Since mitochondrial proteins BCL2/adenovirus E1B 19kDa interacting protein 3 (BNIP3), NIP3-like protein X (NIX/BNIP3L) and FUN14 domain containing 1 (FUNDC1) can directly interact with LC3-II, it is thought that other mechanisms exist to enhance mitophagy (66-69). So far we have discussed how mitochondria play a crucial role in NLRP3 inflammasome activation, but not many studies have been performed about detailed molecular mechanisms of mitophagy. Recently, some studies showed how mitophagy is regulated to control NLRP3 inflammasome activation. Zhong *et al*. found that a cargo receptor p62 is increased by NF-Kb signaling, and the increased p62 is translocated to damaged mitochondria which are ubiquitinated by Parkin, and induces mitophagy (70). Consistent with this finding, Kim *et al*. also observed that p62 is increased and translocated to damaged mitochondria in NLRP3 inflammasome activated cells. Not only p62 but also autophagic inducer Sestrin 2 (SESN2) is increased by NO and translocated to damaged mitochondria and protect cells from hyperinflammation inducing mitophagy. Additionally, SESN2 increases ULK1 stability leading to initiation of autophagy (71). These studies indicate that mitophagy is one of self-limiting systems to protect cells from excessive

inflammation.

CONCLUSION

In the past decade, there have been great advances in understanding of NLRP3 inflammasome and autophagy. However, knowledge about the link between the NLRP3 inflammasome and autophagy, especially mitophagy has been poorly understood. Nevertheless we discussed about the role of mitophagy as a negative regulator of aberrant NLRP3 inflammasome activation, detailed mechanisms are largely unknown. There are questions remained to be answered. Mitochondrial damage can activates NLRP3 inflammasome but inflammatory cytokines also can trigger mitochondrial damage, so it is ambiguous as to which one comes first. Since NLRP3 inflammasome activation has a beneficial effect so how to regulate the activation appropriately should be considered to develop for clinical application. Also, how damaged mitochondria serve ‘eat-me’ signals in the case PINK1 is not mediated should be discovered. Further studies are required for better understanding of molecular mechanism of mitophagy but mitochondria is still a possible therapeutic target to protect cells from aberrant inflammation.

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FIGURE LETENDS

Figure 1. Mitophagy downregulates aberrant NLRP3 inflammasome activation. NLRP3 inflammasome stimuli activates NF-κB producing NLRP3 and pro-IL-1β. Mitochondria is damaged by NLRP3 inflammaosme stimuli and release NLRP3 inflammasome activating signals such as mtDNA and mtROS triggering inflammatory response. Mitophagy downregulates NLRP3 inflammasome activation by elimination of damaged mitochondria blocking NLRP3 inflammasome activating signals.

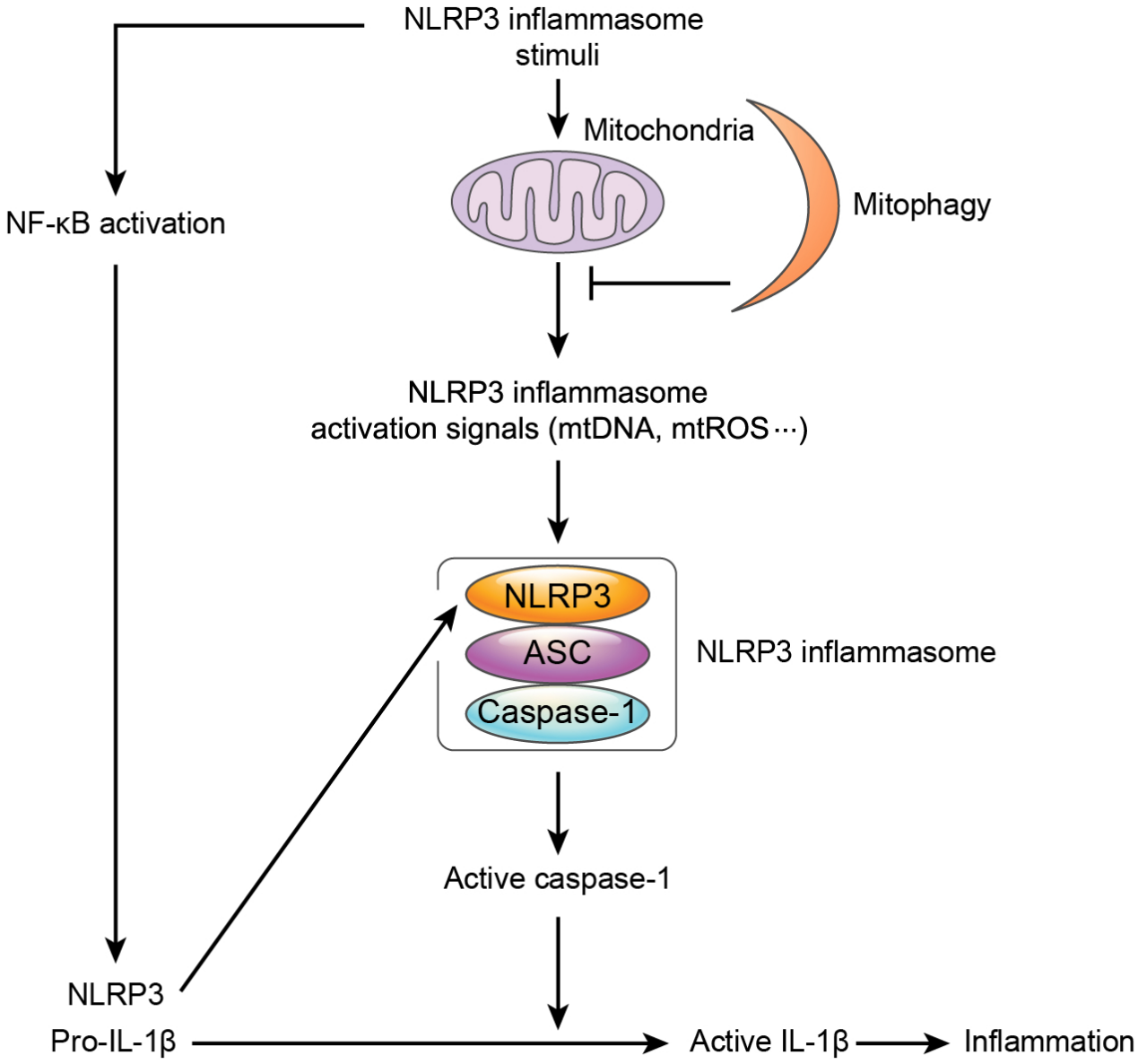
NLRP3 inflammasome
stimuli
Mitochondria
Mitophagy
NF-κB activation
NLRP3 inflammasome
activation signals (mtDNA, mtROS···)
NLRP3
ASC
Caspase-1
NLRP3 inflammasome
Active caspase-1
NLRP3
Pro-IL-1β
Active IL-1β
Inflammation

Fig. 1