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Calpain-1 Mediated Mitochondria ROS/NLRP3 Inflammasome in Atherosclerosis

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Abstract

Calpains are calcium-activated cysteine proteases. There are two main isoforms of calpain that are ubiquitously expressed in tissues, calpain μ or calpain 1, which requires micromolar Ca^{2+} for activation, and calpain or 2, which requires millimolar Ca^{2+} for activation. The presence of other calpains is tissue specific. Atherosclerosis (AS) is an important risk factor for cerebral infarction, coronary heart disease and peripheral vascular disease. It was originally thought that AS was caused by impaired lipid metabolism. This research briefly reviewed Calpain Family, the structure and activation mechanism of calpain1, Calpains in the pathogenesis of atherosclerosis, NLRP3 structural characteristics and activation, ROS/NLRP3 inflammasome activation mechanism and ROS/NLRP3 inflammasome in atherosclerosis. The research showed that the Calpain-1 may play an important role in mitochondrial ROS/NLRP3 inflammasome in atherosclerosis.

Keywords

Calpain-1, ROS/NLRP3 Inflammasome, Atherosclerosis (AS)

1. Introduction

Atherosclerosis (AS) is an important risk factor for cerebral infarction, coronary heart disease and peripheral vascular disease [1]. It was originally thought that AS was caused by impaired lipid metabolism [2]. In 2011, Hansson *et al.* [3] proposed that the inflammatory process and lipid metabolism jointly promote the formation of AS plaques in the arterial wall. However, recent studies have shown that the inflammatory response is crucial in the development of AS. A large number of inflammatory cell infiltrations dominated by macrophages have been detected in AS plaques, and the degree of infiltration is closely related to the severity of the disease [4]. In addition, the levels of inflammatory cytokines

and chemokines are up-regulated in the whole process of AS [5], and the pro-inflammatory factors interleukin (IL)-1β and IL-18 play an important role in the whole process of AS. Its secretion level is positively correlated with the severity of cardiovascular events triggered by AS [6]. To study the role of NOD-like receptor pyrin domain containing 3 (NLRP3) inflammasome in AS will help to provide new research ideas and development directions for the anti-inflammatory treatment of AS.

2. Calpain Family

Calpains are calcium-activated cysteine proteases, there are two main isoforms of calpain that are ubiquitously expressed in tissues, calpain μ or calpain 1, which requires micromolar Ca^{2+} for activation, the presence of other calpains is tissue specific. For example, calpain-3 is a skeletal muscle-specific protease, although its expression is transient in the human embryonic heart leading to recessive limb-girdle muscular dystrophy [7] [8] [9]. In many cases, it is difficult to identify the functional differences of all calpains. Calpain-1 and calpain-2 are both heterodimers with an identical regulatory subunit, calpain4/CAPNS1. The interaction between Ca^{2+} and calpain-1 and calpain-2 induces the functional domain to activate the enzyme activity [10]. In the absence of cytosolic calcium flux, calpain can be activated by direct phosphorylation at serine 50 by extracellular signal-regulated kinase (ERK). Interestingly, during myocardial ischemia, calcium overload induces calpain translocation to the sarcolemma, but intracellular acidosis inhibits calpain activation. Calpain activity is tightly regulated by calpastatin, a specific endogenous calpain inhibitor that regulates specific substrates for proteolysis. These targeting substances are considered intracellular when they are expressed in the cytoplasm. The structural determinants of calpain cleavage substrates are not well established, several of which (e.g. the NF-κB complex and the ATP-binding cassette transporter A1, ABCA1) are rich in sequences of proline, glutamate, serine and threonine amino acid (PEST domain), enhances calpain binding and calpain-dependent proteolysis [11]. It appears that many calpain substrate proteins are also targets of MMP-2 degradation, suggesting that the two proteases share the same substrate sequence, or because the identification of protein substrates for calpain and MMP-2 was based on non-selective calpain inhibitors (*i.e.* can affect MMP-2 and also inhibit the effect of calpain), the efficiency of calpain may be overestimated. Nevertheless, after calpain 1 knockout in mice, it was found that calpain-1 only maintains the essential functions of normal platelets [12], while homozygous calpain 2 knockout mice will lead to embryonic death at the morula and blastocyst stages. The results indicated that calpain-2 is necessary for embryonic development, and calpain-1 and calpain-2 have different physiological functions. In addition, when the regulatory subunit capns1 was knocked out, the activities of both calpain-1 and calpain-2 were inhibited, and at E10.5 days, embryos showed defects, especially in the cardiovascular system, and at E11.5 days, embryos were lethal [13]. Isolation of fibroblasts

from these mice revealed that calpain plays physiological roles in cell migration, cytoskeleton composition, apoptosis, autophagy, plasma membrane repair, and membrane vesicles [14]. Transgenic mice conditionally overexpressing capn1 have increased intracardiac proteolytic activity and increased ubiquitination and protease activity of cardiac proteins under unstressed conditions. In contrast, transgenic mice overexpressing the calpastatin inhibitor calpastatin significantly reduced cardiac ubiquitination of cardiac proteins, causing progressive dilated cardiomyopathy, the pathological change of which is the accumulation of specific non-ubiquitinated proteins, triggering autonomic the phagocytosis destruction pathway is activated [15]. Calpains are primarily located intracellularly, but some of these proteases can be detected outside of tissue cells. In fact, the active secretion of calpains is independent of cell destruction, and the molecular mechanisms by which they are secreted and their functions are being investigated. Calpain can be secreted by lymphocytes, endothelial cells, chondrocytes and osteoblasts, as well as other cells [16]. This mode of secretion is considered an unconventional one due to the lack of an N-terminal canonical secretion signal peptide. However, in addition to being present on the cytoplasmic side of the endoplasmic reticulum and Golgi apparatus, calpain is also contained in their lumen and is eventually secreted [17] or calpain can be secreted to the membrane microspheres of lymphocytes, endothelial cells and parathyroid cells in the bubble. Serine phosphorylation by ERK and protein kinase C may be required for calpain secretion, while the calpain phosphatase protein phosphatase 2A (PP2A), reduces calpain secretion. The extracellular role of Calpain is still unclear. Mainly include can damage intracellular proteins, promote cell migration and invasion. Interestingly, a new hypothesis proposes that unconventionally secreted calpain provides a mechanism whereby calpain activity functions differently inside and outside the cell depending on its intracellular or extracellular localization [18].

3. Calpains in the Pathogenesis of Atherosclerosis

Earlier studies showed that low-density lipoprotein and oxidized low-density lipoprotein promote calpain-2 expression in endothelial cells of atherosclerotic plaques [19] [20]. Activated calpain directly cleaves VE-cadherin and promotes the extravasation of inflammatory cells and macromolecules into the vessel wall [21]. Cytokines and inflammatory signals are involved in the development of atherosclerosis. Nuclear factor-κB (NF-κB) signaling is a representative inflammatory signal, and calpain relieves the phosphorylation of NF-κB by degrading IκB. Calpain inhibitor can significantly reduce the levels of NF-κB and cytokines in endothelial cells [19]. In smooth muscle cells, activation of calpain is thought to precede activation of collagen degradation by caspase, and inhibition of calpain reduces the apoptotic response, suggesting a potential role for calpain in atherosclerotic plaque rupture [22]. Therefore, calpain can activate NF-κB in endothelial cells and smooth muscle cells, and promote the inflammatory re-

sponse. ABCA1 can remove cholesterol by participating in the esterification process of high-density lipoprotein. Calpain-1 can degrade ABCA1, so it is considered to be one of the mechanisms of atherosclerosis. Endothelial cell-specific expression of calpain-2 can cause atherosclerotic lesions by modifying low-density lipoprotein. Calpain-2 can shear the cadherin of the vascular endothelium, resulting in the disintegration of the adhesion junctions between cells and the increase in the permeability between the vascular endothelium [19].

4. NLRP3 Structural Characteristics and Activation

The NLRP3 inflammasome is mainly located in the cytoplasm, and its main structure includes three parts: the core protein NLRP3, the apoptotic speck protein containing a CARD (ASC) and the effector protein cysteine aspartate specific protein 1 (caspase-1). The activation process of NLRP3 inflammasome is tightly regulated by the body, and its activation generally requires two parts of signals-initiating signal and activation signal [23]. Priming refers to the binding of various pathogen-associated molecular patterns (PAMPs), damage-associated molecular patterns (DAMPs) to pattern recognition receptors (PRRs), or after cytokines (TNF, IL-1β) are combined with their corresponding receptors, on the one hand, the inflammatory response signal transduction pathway is activated, NF-κB activation, the transcriptional expression of inflammasome components NLRP3, caspase1 and pro-IL-1β upregulated [24]. On the other hand, the initiation signal activates the post-translational modification of NLRP3. Although the modified NLRP3 is not activated, it is in a highly reactive state. Once it receives the activated second signal, it can rapidly activate the inflammasome and mediate the inflammatory response [25]. After the cells that have received the first signal are activated by the second signal, the NLRP3 inflammasome is rapidly activated to form active caspase1 to cleave pro-IL-1β and pro-IL-18 and release IL-1β and IL-18. In addition, activated caspase1 cleaves intracellular gasderminD (GSDMD) to generate GSDMDN-terminus. The GSDMDN-terminus binds to phosphatidylinositol phosphate and phosphatidylserine contained in the cell membrane to form a 10 - 14 nm hole in the cell membrane, mediating Pyroptosis, while releasing IL-1β, IL-18 [26].

5. ROS/NLRP3 Inflammasome Activation Mechanism

Studies have shown that a variety of NLRP3 inflammasome activators can lead to mitochondrial dysfunction and ROS release, thereby activating the NLRP3 inflammasome, while ROS scavengers can inhibit the activation of the NLRP3 inflammasome. Mitochondria are organelles that provide energy to cells by generating ATP through aerobic metabolism. As a by-product of oxidative phosphorylation, mitochondria continue to produce cellular reactive oxygen species (mtROS), and during cellular stress, the level of mtROS increases. Significantly increased, its entry into the cytoplasm activates the NLRP3 inflammasome [27]. Mitophagy suppresses NLRP3 inflammasome activation by removing damaged and dysfunctional mitochondria, reducing mtROS, and using mitophagy inhibi-

tors to promote NLRP3 inflammasome activation [28]. Imiquimod, a small molecule adenine derivative, activates the NLRP3 inflammasome by inhibiting quinone oxidoreductase and mitochondrial complex I [29]. Its activation depends only on mtROS and is not related to K^+ efflux and lysosomal destruction, which is consistent with other studies using inhibitors of mitochondrial complexes I and III to induce mtROS, thereby activating the NLRP3 inflammasome [30]. Nuclear factor-E2-related factor 2 (Nuclear factor erythroid derived 2-like2, Nrf2) is the main regulator of the body's antioxidant defense system. On the one hand, it inhibits ROS generation and NLRP3 activation by inducing an increase in antioxidant gene expression [31]. On the other hand, by inhibiting the activation of NF-κB, reducing the expression of NLRP3, CASP1, IL-1β and IL-18, and reducing the activity of the NLRP3 inflammasome [32].

6. ROS/NLRP3 Inflammasome in Atherosclerosis

Atherosclerosis (AS) is a disease characterized by the destruction of the integrity of vascular endothelial cells and the proliferation of smooth muscle cells and fibroblasts. Its main feature is the formation of lipid atheromatous plaques under the intima of large and medium arteries, and the specific mechanism is currently unclear. Recent studies have shown that high-risk factors such as hyperlipidemia, hypertension, smoking, and diabetes can increase lipid peroxidative damage in body cells and promote the formation and development of AS [33]. The occurrence and development of AS caused by the above-mentioned high-risk factors are closely related to the abnormality of endovascular factors (plasma lipoproteins, platelets and hemorheology) and vascular wall factors (endothelial, smooth muscle and mononuclear macrophages). 1) Endovascular factors: Low-density lipoprotein is the main factor that causes endothelial and smooth muscle injury, and endothelial injury is considered to be the initiating link of AS. ROS can inhibit the activity of prostacyclin (PGI2) synthase and promote the synthesis of thromboxane A2 (TXA2), which reduces the ratio of PGI2/TXA2, leading to platelet aggregation and thrombosis. ROS also increases blood viscosity by increasing lipid peroxides, leading to cross-linking of red blood cells and plasma proteins; 2) Vascular wall factors: ROS damages endothelial cells, weakens the intimal barrier, infiltrates blood lipids, and activates endothelial cells at the same time, release endothelin and express adhesion molecules. After being activated by ROS, monocytes can induce the expression of various cytokines such as tumor necrosis factor and interleukin through the activation of nuclear factors. Cytokines induce macrophages and endothelial cells to enter the endothelial layer, and oxidized low-density lipoproteins are recognized by scavenger receptors on macrophage membranes for uptake that is not regulated by negative feedback. With excessive lipid accumulation, smooth muscle cells migrate and proliferate under the indirect action of ROS, forming foam cells, which eventually lead to the formation of AS. Recently, a large number of clinical and animal experiments have suggested that ROS generated by NAD(P)H oxidase plays a key role in the pathogenesis of AS. This NAD(P) oxidase is called vascular NAD(P)H

oxidase (partially different from the subunit of macrophage NAD(P)H oxidase), which refers to vascular smooth muscle cells, endothelial cells and adventitia NAD(P)H oxidase in fibroblasts [34]. Recently, Azumi *et al.* [35] reported that in patients with coronary atherosclerosis, ROS production and oxLDL were spatially related to the subunit p22phox of NAD(P)H oxidase. This suggests that ROS catalyzes the formation of oxLDL, leading to the phagocytosis of oxLDL by macrophages to form activated foam cells [36]. In addition, studies have confirmed that ROS products are highly expressed in unstable angina compared with stable angina, suggesting that ROS may also be involved in regulating plaque stability. ROS-induced expression of matrix-degrading enzymes such as matrix metalloproteinases MMP2 and MMP9 initiates plaque instability [37].

Endothelial cell dysfunction is a key link in the formation of AS. When endothelial cells are disturbed by various damage factors, the damaged endothelial cells further release active substances and adhesion factors to mediate the occurrence and development of AS. [38] found that ox-LDL up-regulated the expression levels of NLRP3, caspase-1, and IL-1β by activating endothelial cell apoptosis signal-regulating kinase 1 (ASK1). Ox-LDL significantly increased the expression levels of caspase-1, IL-1β and lactate dehydrogenase (LDH) by inducing the overexpression of MLKL in endothelial cells, which could be blocked by MCC950 [39]. Bian *et al.* [40] proposed that C-reactive protein (CRP) promotes the expression of IL-1β precursor and NLRP3 through the FcγRs/NF-κB pathway. In addition, CRP can also upregulate ROS and promote the activity of caspase-1. The NLRP3 inflammasome is activated to release IL-1β. Sun *et al.* [41] pointed out that trimethylamineN-oxide (TMAO) activates the NLRP3 inflammasome through ROS-TXNIP to induce endothelial cell dysfunction. On this basis, studies have found that apigenin can effectively inhibit the expression of TMAO on intercellular cell adhesion molecule (lintercellular cell adhesion molecule-1, ICAM-1), vascular cell adhesion molecule-1 (vascular cell adhesion molecule-1, VCAM-1) and NLRP3 protein expression and suggested that apigenin has the effect of preventing AS [42]. TNF-α can induce the up-regulation of the expression levels of NLRP3 and IL-1β in smooth muscle cells, and when NLRP3 is inhibited, the secretion level of IL-1β decreases, suggesting that the smooth muscle cell NLRP3 inflammasome may be a potential target for the treatment of AS [43]. Nicotine is an important risk factor for the induction of AS. Yao *et al.* [44] found that rosmarinic acid could inhibit the activation of the NLRP3 inflammasome in smooth muscle cells induced by nicotine and delay the progression of AS. A recent study found that the caspase-1 inhibitor VX-765 inhibited ox-LDL-induced NLRP3 inflammasome activation in smooth muscle cells to control AS progression [45].

7. Conclusion

Studies have showed that activated calpain directly cleaves VE-cadherin and promotes the extravasation of inflammatory cells and macromolecules into the vessel wall. Cytokines and inflammatory signals are involved in the development

of atherosclerosis. AS is a chronic inflammatory disease caused by multiple complex factors. The production of inflammatory cytokines is the key to the development of its disease course. NLRP3 inflammasome and its underlying pro-inflammatory factor IL-1β are closely related to AS, and inhibiting the expression of NLRP3 inflammasome can effectively prevent AS progression and reduce the risk of plaque rupture. Further exploration of the pathogenic mechanism of NLRP3 inflammasome in AS will provide a theoretical basis for the development of AS-targeted drugs. NLRP3-related signaling pathways and the inflammatory cytokines produced by them may become new targets for AS treatment.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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