Mini-review

Role and mechanism of ROS scavengers in alleviating NLRP3-mediated inflammation

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Abstract:

Inflammation, as a common immune response to various infections or injuries, can cause many dangerous and complicated diseases. Inflammasome is a protein complex playing a vital role in an inflammation process, and the nucleotide-binding oligomerization domain (NOD)-like receptor containing pyrin domain 3 (NLRP3) inflammasome has been the most-widely studied one. Recent evidences suggest the reactive oxygen species (ROS)-NLRP3 signaling pathway be a possible NLRP3 inflammasome regulation model. Numerous recent pre-clinical reports indicate that application of antioxidants could scavenge excessive ROS and attenuate inflammatory responses through suppressing NLRP3 inflammasome activation. This article at
first briefly overviews how ROS may mediate the regulation of NLRP3 inflammasome activation. Then, pre-clinical researches of various ROS scavengers for treating NLRP3 inflammasome-associated diseases are focused on and critically analyzed. Finally the potential of antioxidant treatment as a therapy for inflammation is to be discussed, and perspectives on future research directions will be shared.

**Key words:** inflammation, NLRP3 inflammasome, antioxidant biotechnology, ROS, signal transduction pathway

**Inflammation, NLRP3 Inflammasome and ROS**

Inflammation, as a very common and vital immune response to infections or injuries, occurs both in human beings and animals, such as alteration, exudation, and proliferation at a specific inflammatory tissue, then fever, pain and dysfunction locally or even systemically. Inflammation has been reported to be closely linked with many dangerous diseases besides common inflammatory diseases, including neurodegenerative diseases [1], cancer [2] and cardiovascular diseases [3].

A type of multi-protein oligomers called ‘inflammasome’ was found playing an important role in inflammatory response, when the pathogenesis of inflammation was studied [4]. Many researches have confirmed that inflammasomes promote the maturation and secretion of pro-inflammatory cytokines such as interleukin-1β (IL-1β) and interleukin-18 (IL-18) [5, 6].
The nucleotide-binding oligomerization domain (NOD)-like receptor containing pyrin domain 3 (NLRP3, also known as NALP3, cryopyrin) inflammasome has been one of the most-widely studied inflammasomes to date. It belongs to a subfamily of the nucleotide-binding domain and leucine-rich repeat (LRR)-containing family (NLR family), the NLRPs. Once NLRP3 inflammasome is assembled and activated, it will activate caspase-1 by proteolytic cleavage, which will convert pro-IL-1β into bioactive IL-1β, leading to inflammatory responses in bodies. Despite the specific regulatory mechanism of NLRP3 inflammasome activation still remains unclear, reactive oxygen species (ROS) has been frequently reported to be correlated with NLRP3 inflammasome activation.

ROS is well-known as a kind of normal metabolic products of redox reactions. The normal balance of ROS could bidirectionally regulate cell apoptosis and proliferation, and also activate transcription factors, being necessary for a series of signal transduction pathways. The excessive level of ROS would damage the integrity of cells, result in dysfunctions of tissues by causing peroxidation of lipids, proteins, mitochondria and DNA of cells. If the dynamic balance of ROS could be well maintained by internal ROS-scavenging systems or external antioxidants, the ageing process and the ROS-related pathological process might be effectively alleviated or restrained.

This review article is to summarize the recent advances of relationship between ROS and regulation of NLRP3 inflammasome. The focus will be put on the therapeutic potential of
various antioxidants to mitigate inflammation through suppressing NLRP3 inflammasome activation.

**Role of ROS in NLRP3 Inflammasome Regulation**

It has been controversial for a long time whether ROS is indispensable for the whole NLRP3 inflammasome activation process [7-10]. In the following, some typical conclusions from different groups are introduced.

A two-signal model for the NLRP3 inflammasome regulation is commonly recognized [11-13]. Signal 1 manner through NF-κB signaling pathway, which can be affected by ROS, is responsible for the priming step of NLRP3 inflammasome by regulating the expression of NLRP3 and pro-IL-1β [11, 12]. Signal 2 manner is far more complicated and considered to regulate the activation of NLRP3 inflammasome. It is usually classified into two independent pathways, a non-canonical NLRP3 inflammasome activation pathway dependent on Caspase-11 activation and a canonical NLRP3 inflammasome activation pathway without Caspase-11 participation [7, 12, 14]. The non-canonical NLRP3 inflammasome activation is induced by Gram-negative bacteria, while canonical activation could be initiated by various stimuli including Gram-positive bacteria [14]. Pro-Caspase-1 would cleave into activated caspase-1 during both canonical and non-canonical NLRP3 inflammasome activation process, and activated Caspase-1 would convert pro-IL-1β and pro-IL-18 into mature IL-1β and IL-18...
Different from Caspase-1, Caspase-11 could only induce pyroptosis but not process pro-IL-1β or pro-IL-18 (Figure 1) [14, 15].

Although ROS cannot be declared necessary in both canonical and non-canonical NLRP3 inflammasome activation pathways, it could be regarded as a critical second messenger in NLRP3 inflammasome activation process. ROS has already been detected to be deeply involved in many researches on NLRP3 activation triggered by various stimuli [11, 14-17]. In addition, it is also reported that ROS could activate NLRP3 inflammasome through regulating Caspase-11 expression [7], but the necessity of ROS in Caspase-11 regulation requires more direct evidences. It is widely accepted that NF-κB signaling is responsible for initiating NLRP3 inflammasome at transcriptional level [11, 12, 18, 19]. Evidences demonstrate that NLRP3 expression could be enhanced when NF-κB protein level is elevated [20-22]. Considering the in-depth relationship between ROS and NF-κB signaling pathway [23, 24], it is reasonably assumed that ROS may regulate the NLRP3 inflammasome priming through NF-κB signaling pathway. The positive correlation between NF-κ B and NLRP3 has been detected both in vivo and in vitro, and various antioxidants have been reported to suppress the NF-κ B/NLRP3 pathway [25-28]. However, one group claimed that NLRP3 inflammasome activation could be attenuated by IL-4 through a non-transcriptional regulation manner, independent of TLR/NF-κB signaling or mitochondrial ROS [29]. Therefore, more evidences are required to confirm whether ROS is really indispensable in regulating NLRP3 expression or not. If simplex inhibition of ROS production could significantly block NLRP3 expression
expression induced by different stimuli in different cell models without affecting other NF-κB regulators, then the necessity of ROS could be verified.

Also, many evidences support the opinion that ROS contributes to activating the NLRP3 inflammasome. As mentioned above, it was reported that ROS production enhanced Caspase-11 expression and activation, then activated NLRP3 in a mouse model of enteropathogenic *Citrobacter rodentium* infection [7]. However, the link between ROS and Caspase-11 is still not very clear.

A lot more results are consistent with the viewpoint that ROS plays an important role in NLRP3 inflammasome activation. Mouse models of different diseases, including intestinal inflammatory diseases and cardiovascular diseases, have confirmed that ROS regulated NLRP3 inflammasome expression and/or activation [7, 30-32]. The results of many *in vitro* experiments were in good agreement with the above conclusion [33, 34]. Although the specific regulatory mechanism of NLRP3 inflammasome activation by ROS is still elusive, mitochondria were pointed out to be closely related with the activation process.

As known, mitochondria constitute a major source of cellular ROS through mitochondrial respiratory chain. To generate an $H^+$ gradient that could supply the power for ATP synthesis, $O_2$ is reduced to $H_2O$ at mitochondria, while several types of ROS, like $O_2^-$ and $H_2O_2$, are formed at the meantime. Zhou *et al.* conducted a series of experiments and directly proved that the NLRP3 inflammasome activation relied on mitochondrial ROS (mtROS)
generated from mitochondrial respiratory chain [8]. More reports indicated mitochondrial dysfunction and mtROS mediated NLRP3 inflammasome activation [35-39], while mtROS generation was not affected by deficiency in NLRP3 [40]. On the other hand, mtROS generation induced by ATP could result in oxidized mtDNA and mitochondrial dysfunction, which was also reported to promote NLRP3 inflammasome activation [17, 37]. But the oxidized mtDNA was not necessarily the upstream of NLRP3 inflammasome activation [40].

Thioredoxin-interacting protein (TXNIP) is the most studied and discussed protein linking ROS and NLRP3 inflammasome in literature [8, 41-43]. Several substances capable of inhibiting TXNIP induction suppressed endoplasmic reticulum stress-associated TXNIP/NLRP3 inflammasome activation through an AMPK-dependent manner, according to the results from a series of experiments on endothelial cells [44-46]. Repression of TXNIP by antioxidants led to the reduction of NLRP3-mediated inflammation caused by different stimuli as well [46-49]. To elaborate how ROS participated in TXNIP/NLRP3 pathway particularly, a model was proposed earlier [8]. When cells are stimulated and excessive ROS are generated, TXNIP will be released from thioredoxin1 (TRX1) after oxidation by ROS, then TXNIP will bind with NLRP3 inflammasome to activate the latter [8, 50]. The function of TXNIP in the mitochondria is akin. It shuttles from the nucleus to mitochondria under oxidative stress [51], and inhibits reductive properties of mitochondrial TRX2, allowing the accumulation of ROS, until TXNIP shifts to the cytoplasm to interact with NLRP3 later on [52]. The potential close relationship between ROS, TXNIP and NLRP3 inflammasome is partly well explained in the
abovementioned mechanism. Other possible mechanisms like CXCR4/TXNIP/NLRP3 [53], and ER stress/TXNIP/NLRP3 pathway were also proposed [48], but more molecular investigations and complete elucidations are required.

The mitochondrial antiviral signaling protein (MAVS), an outer mitochondrial membrane-associated protein, is another identified mitochondria-associated protein possibly related with NLRP3 activation. In 2013, MAVS was shown to interact specifically with the NLRP3 inflammasome but not interferon-inducible protein AIM2 (absent in melanoma2), in immunoprecipitation experiments using transfected 293T cells [54]. MAVS was found to contribute to NLRP3 localization to the mitochondria and the following NLRP3 oligomerization step in response to ROS [54-56]. However, it is also reported that MAVS could only respond to viral NLRP3 inducers [55], and there have been few antioxidants capable of intervening NLRP3-related inflammation target MAVS proteins until now.

In spite of different results indicating the involvement of ROS in the NLRP3 inflammasome activation [10, 57, 58], one or two essential secondary messengers like ROS intermediating NLRP3 inflammasome regulation were supposed to be necessary to respond to a variety of exogenous and endogenous stimulatory signals. Despite the exact complete regulatory mechanism of NLRP3 inflammasome has not yet been revealed, possible aforementioned mechanistic models of ROS-NLRP3 pathway could help explain the broad connection between oxidative stress and inflammation [15]. What is more, plenty of
Antioxidants have already shown their capability of alleviating NLRP3-inflammasome-associated inflammation by scavenging ROS, which are to be discussed in several important diseases as follows.

**Treatment of Antioxidants for Suppressing NLRP3 Inflammasome-Related Diseases**

**Antioxidants for cardiovascular diseases**

Cardiovascular disease is recognized as the most dangerous one causing people death in the world. Its etiology is usually linked with vascular inflammation and aberrant hemodynamic changes. Role of NLRP3 inflammasome in cardiovascular disease has frequently been reported. The activation of NLRP3 inflammasome was verified to contribute to hyperhomocysteinemia (HHcy)-aggravated inflammation and atherosclerosis in apoE−/− mice [59]. In this high fat plus high methionine diet-induced HHcy mice model, the necessity of intracellular ROS participating in NLRP3 inflammasome activation was confirmed. Simultaneously, intervention with an efficient antioxidant N-acetyl-L-cysteine (NAC) obviously mitigated HHcy-induced NLRP3 inflammasome activation and atherosclerotic lesion formation [59]. NAC also inhibited the ROS-mediated nicotine-NLRP3-ASC-pyroptosis pathway and prevented endothelial cell pyroptosis [60]. Oxidative stress modulation of NLRP3 inflammasome activation in cardiovascular disease was also reported in a very recent case [61]. After high-salt or low-salt treatment, the change of insulin resistance index was found notably related with NLRP3 inflammasome activation both
in vitro and in vivo. Administration of NAC effectively prevented the NLRP3 inflammasome activation induced by high salt in THP-1 cells. Rosuvastatin, a member of 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors, significantly inhibited NLRP3 inflammasome activation in an isoproterenol-induced myocardial infarction injury rat model by attenuating oxidative stress [30]. Flavonoid is a big subfamily of polyphenols with free radical scavenging capacity and antioxidant activity, and different flavonoids could scavenge excessive ROS and subsequently protect against endothelial senescence via ROS-NLRP3 inflammasome signaling pathway [62]. Similarly, hispidulin was proved to have ameliorative effects on high glucose-mediated endothelial dysfunction by inhibiting NLRP3 inflammasome activation. Results indicated that hispidulin could attenuate PKCβII phosphorylation and the following ROS production, reversing the loss of mitochondria membrane potential caused by high glucose environment [20]. Moreover, an enhanced expression of NLRP3 and IKKβ/NF-κB by a high glucose level was significantly restored after administration of hispidulin. Hispidulin was thus found to prevent endothelial dysfunction through PKCβII-associated NLRP3 inflammasome activation and NF-κB signaling, showing its potential to treat diabetic vascular complications. Peperomin E (PepE), another antioxidant phytochemical, similarly suppressed high-fat-diet (HFD)-induced atherosclerosis in ApoE-/ mice [26]. Increased phosphorylated IκBα and NF-κB protein levels by HFD in artery tissues were both decreased with PepE addition.
TXNIP is also reported to mediate ROS-dependent NLRP3 inflammasome activation in cardiovascular disease-related studies [63, 64]. In a myocardial ischemia/reperfusion injury (MI/R injury) model, interaction between TXNIP and NLRP3 was identified as a key mechanism for NLRP3 activation and MI/R injury in mouse model. *In vitro* experiments revealed that the interaction was relying on ROS generation. An ROS scavenger EUK134 dissociated TXNIP from NLRP3 and blocked the NLRP3 inflammasome activation process in cardiac microvascular endothelial cells (CMECs) [64]. Another work showed the same conclusion, demonstrating that TXNIP binding to NLRP3 with ROS involvement was a key signaling mechanism necessary for HHcy-induced NLRP3 inflammasome formation and activation as well as subsequent glomerular injury [63].

Trimethylamine-N-oxide (TMAO) was recently used to promote atherosclerosis by inducing vascular inflammation [65, 66]. TMAO promoted NLRP3 and caspase-1 expression in human umbilical vein endothelial cells (HUVECs) and apoE<sup>−/−</sup> mice [66]. ROS generation, particularly mtROS, was additionally stimulated by TMAO through inhibiting manganese superoxide dismutase 2 (SOD2) activation and sirtuin 3 (SIRT3) expression. The mtROS scavenger mito-TEMPO was able to reverse the regulation and attenuate vascular inflammation. The necessity of SIRT3 was validated by the fact that TMAO failed to inhibit manganese SOD2 or activate NLRP3 inflammasome to induce inflammation in either SIRT3<sup>−/−</sup> cells or SIRT3<sup>−/−</sup> mice. The proposed SIRT-SOD2-mtROS signaling pathway mediating
NLRP3 inflammasome activation may be another novel therapeutic target of ROS scavengers to intervene ROS-NLRP3-dependent vascular inflammation.

**Antioxidants for brain diseases**

Inflammation occurring in cerebral cells is one of the main causes of brain diseases. Since NLRP3 inflammasome activation is a key process of such inflammation, inhibitors of this signaling process have the potential to prevent and treat various brain diseases through scavenging ROS [1]. An animal model of subarachnoid hemorrhage (SAH) is a common model studying early brain injury (EBI). Melatonin was observed to remove ROS and attenuate neuronal inflammatory response after SAH. As a powerful endogenous antioxidant, melatonin can directly neutralize dangerous free radicals, and indirectly scavenge ROS via inducing activation of antioxidant enzymes [67]. Recently, mitophagy was considered a key reason why melatonin was neuroprotective against EBI in a study of SAH reported in 2017 [68]. The work demonstrated that melatonin upregulated mitophagy-associated proteins and reduced ROS generation, thus inhibiting NLRP3 inflammasome activation. In a report this year, SIRT3 protein expression enhanced by melatonin was observed to reduce ROS, then inhibit NLRP3 inflammasome activation and protect neuronal cells [69]. SIRT3 protein locates at mitochondria and is associated with mitophagy [70], which may be one main target of melatonin regulating ROS-NLRP3 inflammasome pathway, as illustrated in Figure 2.
In intracerebral hemorrhage (ICH) model, both isoliquiritigenin and silymarin could prevent NLRP3 inflammasome activation and protect against ICH. Both plant-derived natural active materials could promote Nrf2 antioxidant pathway in murine to decrease ROS concentration, negatively regulating NF-κB and NLRP3 expression [21, 71]. Ruscogenin, another plant-derived natural substance, was found to reduce cerebral ischemic injury via blockading NF-κB-mediated inflammatory signaling pathway, but NLRP3 inflammasome was not detected [72]. According to a further research [73], ruscogenin attenuated cerebral ischemia-induced blood-brain barrier dysfunction both in vitro and in vivo. It markedly suppressed NLRP3 activation and downstream pathway by decreasing ROS generation and inhibiting TXNIP/NLRP3 interaction. Thus, it could not be concluded whether ruscogenin was able to suppress NLRP3 inflammasome activation via multiple ROS-related pathways so far, and more studies of application of ruscogenin in treating NLRP3 inflammasome-associated diseases are required.

On the other hand, mitochondria is verified as a potential target to treat brain cognitive deficit. In 2015, a mitochondrion-targeted antioxidant SS-31 was used to treat mice suffering cognitive deficits induced by isoflurane, a general inhalation anesthetic[74]. SS-31 protected the mitochondrial integrity and function through clearing mtROS. At the same time, suppressed NF-κB signaling pathway contributed to the decrease of NLRP3 expression mechanistically. In cell culture and animal models of Parkinson’s disease, mitochondrial impairment was also indicated as a probable causative factor. The neurotoxic pesticide caused...
bioenergetics defects and lysosomal dysfunction in microglia, and also substantially increased mtROS generation as well as IL-1β secretion by activating NLRP3 inflammasome. And this process could be distinctly blocked by mitochondria-targeted antioxidant mito-apocynin [75]. The specific mechanism underlying mtROS-scavenger suppressing NLRP3 inflammasome activation was not reported previously, and in 2017 a novel insight into that mechanism was suggested [76]. Lawana and his colleagues showed that LPS priming and subsequent rotenone stimulation enhanced NLRP3 inflammasome activation and led to mitochondrial dysfunction in microglial cells. The course was elicited in a c-Abl tyrosine kinase-dependent manner and could be effectively ameliorated by a mitochondrial antioxidant mito-TEMPO. The mechanistic studies revealed that c-Ab1 functioned as a proximal signal exacerbating NLRP3-related markers such as mitochondrial dysfunction and NF-κB activation. An ROS/c-Abl/NLRP3 signaling axis was first presented based on experiments on LPS-primed ROT-stimulated microglial cells. It still requires more evidences to confirm the ROS/c-Abl/NLRP3 pathway and relationship between ROS and c-Abl in other NLRP3 inflammasome-dependent inflammation models. Nevertheless, the above finding offered a novel therapeutic strategy for PD treatment and it also supported the conclusion that mitochondrial antioxidants could help attenuate inflammation-related brain diseases by inhibiting NLRP3 inflammasome activation.
Antioxidants for inflammatory diseases in liver, kidney and intestines

Common inflammatory diseases in main organs such as liver, kidney, and intestines are reported. These diseases are also associated with ROS-dependent NLRP3 inflammasome activation.

A new antioxidant multitarget iron chelator M30 significantly attenuated ethanol-induced injury in hepatocytes [77]. It was verified that M30 relieved hepatocyte inflammatory responses and NLRP3 inflammasome activation, though the connection between reduced ROS generation and inhibited NLRP3 inflammasome activation was not uncovered. Other reports may help reveal the potential underlying mechanisms. Asiatic acid was effective in ameliorating hepatic ischemia/reperfusion (I/R) injury by inactivation of Kupffer cells via ROS-related PPARγ/NLRP3 inflammasome signaling pathway [78]. ROS-TXNIP pathway was identified as a probable mechanism for fructose-induced nonalcoholic fatty liver disease, which can be intervened and inhibited by antioxidants [79]. Angiotensin-(1-7) modulated redox balance through regulating NOX4 and Nrf2/ARE pathway and inhibited NLRP3 inflammasome activation in vivo and in vitro. It increased glutathione and nuclear erythroid 2-related factor2 (Nrf2) antioxidant response element (ARE), and also decreased NOX4 protein level and H₂O₂ content in bile duct ligation-induced hepatic fibrosis. The finding was confirmed in hepatic stellate cells [80], and the same team observed similar results in angiotensin II-induced hepatocyte epithelial-mesenchymal transition (EMT). The NAC
treatment inhibited hepatocyte EMT, indicating antioxidants were potential therapeutics of this ROS-dependent inflammation process [81].

Studies on kidney inflammation-related diseases had similar findings to the aforementioned ones. Citral protected against accelerated and severe lupus nephritis (ASLN) by inhibiting activation signal of NLRP3 inflammasome. Citral cleared ROS and COX2 production, and also augmented Nrf2 activation in an LPS-induced mice ASLN model [16]. According to in vivo and in vitro tests reported by Shazad et al. [82], minocycline could also stabilize endogenous Nrf2 by scavenging ROS to attenuate NLRP3-inflammasome induced diabetic nephropathy.

The link between ROS-mediated TXNIP and NLRP3 inflammasome activation is another key blockade target to inhibit NLRP3-related kidney inflammation [53, 83]. Gene expression analyses demonstrated that genes encoding for TXNIP, NLRP3, Caspase-1, IL-1β and IL-18 were significantly upregulated in hydroxy-L-proline (HLP)-fed rats, but apocynin administration had these genes downregulated in the cortex and medulla respectively. HLP was used to induce hyperoxaluria and calcium oxalate (CaOx) crystal could promote ROS production, which could be cleared by apocynin [83]. The study revealed the close connection between ROS, TXNIP and NLRP3 inflammasome activation. It was also ascertained by a research on troxerutin preventing inflammatory damage in kidney tissues of BDE-47-treated mice [42]. Additionally, the work indicated that C-X-C chemokine ligand 12 receptor 4
(CXCR4) had a direct interaction with TXNIP. The enhanced expression of CXCR4 caused by BDE-47 was suppressed by troxerutin as well. It was not clear whether CXCR4 could be directly mediated by ROS, because activities of inflammatory factors including COX2, iNOS and NF-κB as well as ROS concentration were all reduced after troxerutin treatment on mice [53]. Nevertheless, the findings improved our understanding of ROS/TXNIP/NLRP3 signaling pathway and antioxidants application in treating inflammatory diseases.

Blockade of Nrf2/ARE pathway may be also considered as a therapeutic in intestinal bowel disease. Oral administration of 3-(2-oxo-2-phenylethylidene)-2,3,6,7-tetrahydro-1H-pyrazino-[2,1-a]isoquinolin-4(11bH)-one (compound 1), a novel small molecular activator of Nrf2/ARE, was proved to be effective in inhibiting NLRP3 priming step in a DSS- induced colitis mice model [32]. Dimethyl fumarate treatment gave the same result [31]. ROS/NF-κB/NLRP3 signaling pathway was another mechanism focused on intestinal inflammation treatment. Procyanidin, a member of flavonoids, showed its powerful ability on ROS clearance and suppression of NF-κB signaling in THP-1 macrophages after LPS stimulation. Meanwhile, the expression of NF-κB and NLRP3 was suppressed and the formation of NLRP3 inflammasome was interrupted by procyanidin in a dose-dependent fashion in a DSS-induced mice colitis model [22]. Dandelion extract mitigated inflammatory signaling NF-κB p65 and cyclooxygenase-2 activity induced by LPS in human colonic epithelial cell line HT-29 cells via suppressing ROS [28]. These facts
indicate that NF-κB/NLRP3 pathway is probably the main one that ROS regulated NLRP3 inflammasome at transcription level.

Further discussion on antioxidants for inflammatory disease treatment

Although there are many other reports on different antioxidants applied to various inflammatory diseases, their main action mechanisms are almost similar to the above-mentioned ones. As follows, we intend to further briefly discuss antioxidants for inflammation treatment in another research angle.

Antioxidative phytochemicals are popularly used to treat inflammation in pre-clinical experiments in recent years. These plant-derived extracts usually could exert multiple effects on organisms including ROS scavenging. They are natural products and usually not toxic because they are mostly derived from traditional herbs, fruits and vegetables having been accepted by people for long time. Many of them clear ROS through enhancing antioxidase expression via Nrf2 pathway, thus regulating ROS-mediated NLRP3 inflammasome expression and/or activation. Autophagy, including mitophagy, may also mediate ROS reduction and suppress NLRP3 inflammatory signaling pathway [68, 69, 84]. Activation of autophagy and decreased ROS production was observed when quercetin suppressed NLRP3 inflammasome activation in epithelial cells infected with E. coli O157:H7 [39]. A research on a daidzein derivative X-11-5-27 also demonstrated that autophagy-mediated ROS reduction
contributed to inhibition of NLRP3 inflammasome activation [38]. Decreased ROS production is not the only result of activated autophagy, but mitophagy could clear damaged mitochondria and then reduce ROS generation [85].

Nevertheless, one deficiency of these promising therapeutic ROS scavengers is that the exact working molecular structure and mechanism of these natural antioxidants are unknown. Since NLRP3 inflammasome formation and activation could respond to multifarious stimuli, some metabolites of these phytochemicals may be new inducers of inflammation in human beings. In another aspect, it may be not easy to verify the complete impacts of these metabolites on inflammation through in vitro tests or animal model tests, because of different interactions between cells and diverse metabolic manners.

Another deficiency is that these oxidants usually clear many types of ROS generated from different sources (Figure 3). Broad but not specific ROS elimination by common ROS scavengers like polyphenols or NAC might trigger other problems, for example, fluctuation of oxidative balance, or intervention of other signaling pathways intermediated by ROS [86]. These wide-spectrum ROS scavengers may help relieve systemic inflammation through digestion or blood circulation, but it cannot be concluded that they could particularly target inflammatory tissues without disturbing physiological balance in other healthy tissues. To lower the side effects or elevate the antioxidative action specificity of original bioactive compounds like cinnamaldehyde [87], it is helpful to design artificial derivatives of these ROS
scavengers. Current derivatives might work better as green antioxidants than natural ones [87, 88], or might not [9], so it is essential to first find out specific effective molecular structure part of natural antioxidants, then design more efficient artificial derivatives for more appropriate clinical screening.

Compared with wide-coverage ROS scavengers, mitochondrial targeted antioxidants like Mito-TEMPO and MitoQ might be more promising ROS scavengers used as NLRP3 inflammasome inhibitors. As previously discussed, it is because that mitochondria is probably an essential unit as a reaction platform for ROS to work on TXNIP or other unidentified proteins to mediate NLRP3 inflammasome activation. However, on the other hand, it should also be taken into account that ROS can be derived from a couple of sources in organisms besides mitochondrion, including ER stress, lysosome dysfunction and other enzymatic members like NADPH oxidase, xanthine/xanthine oxidase, cytochrome P450s. Therefore, a single use of mitochondrial antioxidant might not be powerful and effective enough, if mtROS is not the sole intermediate of NLRP3 inflammasome-related inflammation.

Without doubt, more and more studies and findings of antioxidants are required to promote understanding molecular mechanism of ROS-NLRP3 inflammasome regulation. Such researches are directly valuable to treat NLRP3-related inflammatory diseases with antioxidants.

Perspectives
It has been of great interest studying the relationship between ROS and NLRP3 inflammasome activation in recent years, but a fundamental question whether ROS is prerequisite for the process has not been perfectly answered yet, not to mention the complete specified molecular mechanism. It might be a long way for using antioxidants to treat various inflammatory diseases, due to a number of objective obstacles. NLRP3 is sensitive to ROS generation, while it could be induced by many different types of stimuli as well, endogenous or exogenous ones, viral or non-viral ones, natural or artificial ones. And it might be because of two or more different types of NLRP3 inflammasome activation pathways. Similarly, ROS generation could be aroused by several conditions. ROS inhibitors may limit ROS concentration at a restricted level, but ROS generates almost all the time as long as cells are alive. Apoptosis, autophagy and pyroptosis are also related with ROS, making it hard to limit ROS generation. The accuracy of ROS detection technologies are also imperfect so far, considering the very short damping period of ROS. In addition, it is not easy to determine all functions of ROS scavengers on cells or organisms. ROS scavengers may only clear ROS generation, while they might have other undiscovered stimulatory or inhibitory interventions on cells. Different cell lines used in researches is another factor that affects the results and conclusions. Even environment conditions like extremely low-frequency electromagnetic fields are possible to impact on the antioxidant activity of cells [89]. Compared with newly-artificially synthesized antioxidants, natural or endogenous antioxidants might be less risky having unexpected impacts on patients or animals. However, endogenous antioxidants
like melatonin would exert different or even inverse effects on organisms, depending on minimal changes of concentration.

Although there are nonnegligible difficulties in discovering molecular mechanism of ROS-NLRP3 inflammasome regulation, recent findings have shown a promising future using ROS scavengers to treat NLRP3 inflammasome-related inflammatory diseases. These findings cannot be perfectly applied into clinical treatment yet, but some highlighted directions may be expected. Firstly, it might be promising to design and synthesize derivatives of natural antioxidative components. It will be helpful to alleviate side effects and strengthen the therapeutic effects of natural antioxidants. Secondly, artificially processed endogenous ROS scavengers might be another option. Endogenous scavengers are usually highly-efficient and safe like melatonin, while direct extra administration of endogenous antioxidants might break the original physiological balance inside organisms [78]. If processed ROS scavengers could be metabolized safely and smoothly without significant disturbance on endogenous ones, while maintaining the approximatively efficient therapeutic effects, it might be an excellent medicine. Hydrogen-rich saline is a possible one [90]. Novel antioxidant and anti-inflammatory peptides derived from animals might also have potentials to be processed to a moderate medicine for inflammation by clearing excessive ROS [91]. A multi-target therapy is the third research direction. Because the concrete mechanism underlying ROS-NLRP3 inflammasome pathway remains unclear, and there are a number of ROS sources in organisms, treatment targeting plural possible pathways and sources may help attenuate NLRP3-related...
inflammation more effectively. A multitarget antioxidant appropriate for inflammation treatment like iron chelator M30 might be not easy to find, but it is simpler to make a synergy therapy by combining several differently-targeted antioxidants together. Further investigations are certainly demanded before the therapies applied into clinical use, but these studies are bringing new insights into a mature therapy. More discoveries on molecular mechanisms of NLRP3 inflammasome regulation by ROS are much expected, and we hope that clinical inflammation treatment with ROS scavengers may be achieved in near future.

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**Figure legends**

**Figure 1.** A simplified two-signal model of NLRP3 regulation involved with ROS. Signal 1 pathway regulates NLRP3 expression via NF-κB [11, 12]. Signal 2 pathway is responsible for NLEP3 inflammasome activation, through canonical and non-canonical pathway [7, 14]. Non-canonical pathway is dependent on Caspase-11, but canonical one is not. Both priming
and activating steps of NLRP3 inflammasome regulation can be promoted by ROS generation or inhibited by ROS scavengers \[14, 15\]. Activated NLRP3 inflammasome produces caspase-1, then IL-1\(\beta\) and IL-18.

Figure 2. Illustrated mechanism of melatonin attenuating early brain injury (EBI) involved with ROS. Melatonin could directly scavenge ROS, and it can also enhance SIRT3 protein expression at mitochondria membrane to reduce mtROS production \[68, 69\]. In addition, melatonin is also able to activate antioxidases through Nrf2 pathway \[67\]. The secretion of IL-1\(\beta\) and IL-18 by NLRP3 inflammasome activation would be suppressed because of decreased ROS. This process would protect neurons against inflammatory injury triggered by IL-1\(\beta\) and IL-18 \[68\].
Figure 3. Sources of ROS generation and different action mechanisms of various antioxidants. ROS could be generated from different sources including mitochondria, ER stress, lysosomal dysfunction and oxidases [11, 15]. Various ROS scavengers could inhibit ROS production through different manners as briefly shown in the figure [16, 59, 68, 71, 92].