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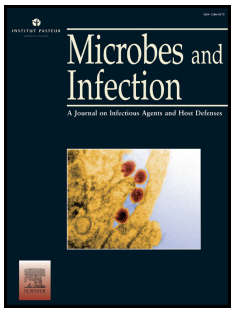

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ABSTRACT: Virus-induced oxidative stress plays an important role in the regulation of the host immune system. In this review, we provide backgrounds of the pathogenic mechanism of oxidative stress induced by influenza virus and the specific oxidant-sensitive pathways, and highlight that antioxidant is one of the effective strategies against influenza virus infection.

KEYWORDS: Influenza virus; Oxidative stress; the immunity system; Antioxidants

1. Introduction

Influenza A virus, belonging to negative sense RNA virus, is one of the major causes of respiratory disease pathogens with characteristics of high morbidity and mortality [1]. Over the past century, the "Flu Pandemic" has repeatedly erupted around the world, including the 1918 Spanish flu, the 1957 Asian flu, the 1968 Hong Kong flu, and the first outbreak of the new influenza H1N1 in Mexico in 2009.The report has shown that the 2009 H1N1 influenza sweep more than 214 countries, resulting in at least 18000 deaths, reaching the highest level 6 of the WHO's Flu Pandemic alert [2]. The recent outbreak of highly pathogenic H5N1 avian influenza possessed a serious threat to human and animals of different species, and the mortality rate was estimated to reach 60% [3-5]. In 2013, a novel recombinant avian influenza virus subtype H7N9 was reported in China, continuing to threat human life [6, 7]. Also, other recombinant avian influenza viruses emerged continuously in the past two years, indicating that we would face unpredicted situation in preventing influenza virus infection in the future [8, 9]. Currently, effective treatments against influenza infection are still inadequate because of current anti-viral drugs resistance and vaccine escape, especially against the highly pathogenic influenza virus [10, 11]. Thus, it is urgent need to develop new antiviral approaches, particularly from host-cell factors essential for viral replication.

Influenza virus as obligate intracellular parasites is dependent on affecting host cell functions to promote its replication. At present, it has found that there are many host-cell factors that can direct or indirect affect viral infection [12, 13].

Pro-/antioxidant balance is critical to maintain normal functioning of host, whereas oxidative stress caused by influenza viral simulation breaks the intracellular redox balance, leading to significant changes of host defense system. For this reason, the establishment of the special environment of the host is very important to resist virus infection. Here, we focus on the role of oxidative stress in influenza pathogenesis and highlight that antioxidant is one of the effective strategies of prophylaxis and treatment of influenza virus infection.

2. Oxidative stress in influenza virus

2.1 Oxidative stress

Oxidative stress (OS), the concept was firstly proposed based on the research of human aging [14]. The widely accepted definition of oxidative stress is an imbalance between oxidants and antioxidants when the organism exposures to adverse stimuli [15].Usually, reactive oxygen species (ROS) are steadily produced under the normal cellular metabolism. Once the accumulation of ROS exceed the removal of the oxide, the balance between the oxidation system and antioxidant system will be broken, resulting in the aggravation of neutrophils inflammatory infiltrates, the rising of protease secretion and accumulation of oxidation intermediate, ultimately causing tissue damage, inflammation response and apoptosis (Fig.1). Emerging evidence has demonstrated that oxidative stress is an important contributor to infectious diseases, such as HBV, HCV, HSV and influenza [16-19].

2.2. Excessive accumulation of ROS enhances influenza virus-induced cell injury

ROS are byproducts of mitochondrial metabolism, including a large variety of

free oxygen radicals, like superoxide anion radical and hydroxyl radical, and highly reactive molecules, such as hydrogen peroxide(H_2O_2) and singlet oxygen (O_2) [20]. Recent reports have shown that ROS exerted a positive or negative effect during influenza virus infection, depending on its production [19, 21]. On the one hand, viral infection can be initiated to produce certain ROS, which play an irreplaceable role in reducing cellular damage. On the other hand, excessive production of ROS make infected cells in a chronic non-acute oxidative stress state, finally accelerating persistently the deterioration of diseases. Excessive ROS, together with its superoxide and derivatives, are main causes of lung injury caused by influenza virus infection. The downstream Nox1 or Nox2 is also relevant to virus-induced epithelial apoptosis and lung injury [22, 23]. Importantly, after invading the host defense system, the influenza A virus will hijack the host cell functions to enhance itself replication [21, 24, 25]. Accordingly, the imbalance of the redox environment is the basis of influenza virus infection and tissue damage. It can also increase susceptibility of the host epithelial cells to influenza virus and promote cell-to-cell viral transmission [26].

2.3 ROS contributes to influenza virus-induced inflammation

As above mentioned, virus invasion may cause oxidative stress, resulting in inflammatory damage. In particular, highly pathogenic influenza virus infections are often accompanied by a dysregulation of and an excessively exaggerated immune response, commonly known as cytokine storm [27, 28].Virus infection induces the robust production of cytokine, including interferons (IFNs), tumor necrosis factors (TNFs), interleukins(ILs) and chemokines, while ROS as an inflammatory mediator

can promotes the expression of these cytokines. Report has indicated that ROS generated by influenza infection leading to the induction of RANTES production[29]. Therefore, ROS might act as a crucial messenger in the process of signal transduction for host immune system. Slightly elevated ROS levels may enhance immune system function, while high levels of ROS could promote a pathological inflammatory response [21].

Pathogen-associated molecular patterns (PAMPS) is recognized by pattern-recognition receptors（PRRS）to generate immune response. It has been shown that the generation of NADPH and ROS were required in activating Toll-like receptors (TLR), RIG-I-like receptors (RLRs), and NOD-like receptors (NLRs), and these pattern-recognition receptors can promote innate immunity to protect host [30, 31]. Furthermore, excessive ROS production can activate the JNK/ERK/p38 MAPK and NF-κB pathways, and these signaling pathways possibly contribute to acute pulmonary damage induced by severe influenza virus infection [32]. Above all, oxidative-stress is another major model that has been used to explain the mechanism of influenza virus-induced immunopathology.

2.4 The relationship between oxidative stress and apoptosis

Cell apoptosis, also named programmed cell death. Apoptosis is triggered by two major pathways, intracellular and extracellular signals, is also called the mitochondrial and death receptor-mediated pathways. The extracellular pathway is mediated by receptors, which is activated by the stimulation of exogenous signaling molecules. The intracellular pathways is regulated by mitochondria mediated

signaling pathway, and the activation of intracellular pathway is related to the non-dependent receptors of ectogenic stimulus and cell endogenous affection, such as DNA damage, oxidative stress[33]. Also, the process of cell apoptosis is involved in a variety of signaling pathways such as, MAPK, TNF, Bcl-2, P53, NF-κB[34].

There are growing evidences showed that oxidative stress is clearly associated with apoptosis in host cells. Previous studies have found that various redox systems such as glutathione and thioredoxin are involved with the cell apoptosis signaling pathway and regulated its internal environment [35, 36] .After influenza virus infected respiratory epithelium, the levels of ROS and NO are more than normal threshold, following with the productions of highly oxidizing nitrogen oxides and peroxynitrite, inducing greatly oxidation or nitration of protein amino acid residues, lipid peroxidation and DNA strand brend break, ultimately generating apoptotic signals in the state of oxidative stress. Thus, oxidative stress is a major mediator of apoptosis induction, and that ROS generation plays a critical role in the mitochondria apoptotic cascade.

3. Oxidative stress pathway

3.1The Nrf2 signaling pathway

Nrf2 (NF-E2-related factor 2) is a high sensitivity transcription factor that regulates cellular antioxidant response. Inactive Nrf2 is recruited by the cytosolic protein Keap1 (Kelch-likeECH-associatedprotein1) under the basal conditions. Next, Nrf2 is rapidly degraded by the ubiquitin proteasome pathway when Keap1 and Cul 3 combined into the E3 ubiquitin ligase complexes [37]. Oxidative stress can drive

dissociation of Nrf2 and Keap1, promoting the translocation of Nrf2 into the nucleus, and subsequent binding to antioxidant response element (ARE) and interacting with transcription factors in the bZIP family (Fig.2A). In the nucleus, Nrf2 activates multiple protective genes expressions which related to antioxidation and Phase II detoxification of xenobiotics, contributing to protect cells against oxidative damage [38]. A recent study confirmed that the production of ROS and the expression of activated Nrf2 were promoted when influenza virus H1N1 infected alveolar epithelial cells. In Nrf2 knockout model, type II alveolar epithelial cells were more sensitive to inflammation and injury. In contrast, expression level of Nrf2 increased and the inflammatory damage of cells reduced when the epithelial cells was infected by adenovirus Nrf2 [39]. Thus, Nrf2-mediated protection of cells from injury during influenza infection is particularly important to cellular antioxidation defense systems.

3.2 The P38 MAPK signaling pathway

Mitogen activated protein kinase (MAPK) is one of the most important signal transduction system. The P38 group MAP kinases belong to MAPK sub-family, which play a vital role in many biological processes. The P38 signaling pathway participates in cellular responses because of a wide range of stimuli, both in vitro and in vivo, and that mediating growth, development, differentiation, and death of cells. For instance, the phosphorylated p38 translocates into the nucleus of host cells and is involved in the expression of cytokine genes under oxidative stress-stimuli (Fig. 2B). Additionally, p38 MAPK signaling pathway was triggered by diverse stimuli to cause acute lung injury (ALI), a common complication in patients with influenza viruses [40].

Therefore, inhibition of p38 MAPK function with its specific inhibitors should be a potential strategy for the prevention or treatment of influenza virus-associated ALI [41].Previous studies have shown that P38 MAPK pathway was sensitive to oxidants and is involved in influenza virus replication [42, 43]. Besides, p38 MAPK pathway is also critical mediators in apoptosis by both generating ROS and up-regulating COX-2 [32].

3.3 The NF-κB signaling pathway

NF-κB as a family of transcription factors has a key regulatory function, especially in the inflammation response. The activity of NF-κB induced by a wide variety of stimuli including TNF-α, PMA, cigarette smoke extract (CSE), LPS, oxidants, and viral infection, so that it has been called a “stress sensor”. A series of studies have advanced our understanding of NF-κB signaling pathway which could be triggered by oxidative stress [44-46].Here, we take the NF-κB P50/p65 heterodimer which is primarily associated with IκBα as an example to elucidate the oxidative stress-induced activation of NF-κB. Under the conditions of oxidative stress evoked by influenza virus, IκBs is phosphorylated by IκB kinase, making IκB separates from P50/p65 heterodimer. Then, the activated NF-κB transfers into the nucleus and binds to specific DNA sequences, leading to follow-up reactions (Fig.2C). In the process of oxidative stress, the NF-κB pathway is activated by excessive ROS and the degree of activation can be regulated by antioxidants. The recent study has indicated that the protective effect of kaempferol on acute lung injury induced by influenza H9N2 virus was related to suppression of the oxidative stress and inflammatory responses by

down-regulation of NF-κB signaling pathway [47]. Inhibition of the NF-κB pathway could confer to efficient protection against lethal influenza challenges. It should be noted that NF-κB pathway and other oxidant-sensitive pathways appear to be activated simultaneously because of excessive levels of ROS stress [48]. For instance, the immunomodulatory properties of NAC which decreased virus-induced oxidant injury in the case of influenza pneumonia was thought to be related to the inhibition of the activation of NF-κB pathway, together with inhibiting the activation of the p38MAPK pathway [49].Overall, detection of NF-κB may serve as a biomarker of oxidative stress and provide new insights into the pathogenesis of oxidative stress caused by influenza virus [50].

4. Antioxidants in anti-influenza virus therapy

4.1 NAC

NAC, one of the common antioxidants, is a precursor of intracellular cysteine and GSH. It plays multiple biological functions, such as antioxidation, anti-apoptosis, interference of free radical production [51]. Reports have shown that NAC controlled influenza virus infection through a number of mechanisms including inhibiting influenza virus replication, reducing production of pro-inflammatory cytokines and preventing virus-induced apoptosis, as described in Table 1[52-56]. Geiler J et al. [54]. reported that NAC could inhibit viral replication, and reduced the expression of influenza virus-induced inflammatory factors like TNF-α、IL-6、IL-1β. Others found that it significantly alleviated the lung damage by inhibiting the levels of TLR4 protein and TLR4 mRNA in the lungs [55]. However, a new argument about antiviral

effect of antioxidants has been put forward. According to Garigliany's study, NAC was obviously strain-dependent for it lacked common inhibitory activity against different influenza A viruses [57] . Unlike the successful results in A/PR/8 and H5N1 strains, NAC could not alter the severe lung injury caused by the murinized swine H1N1 influenza virus in their study. From the point of view, whether NAC is effective for new viruses when it outbreak is still worth discussing. Although the above argument about antiviral effect of antioxidants has been proposed, this cannot deny the fact that NAC has an active effect toward some influenza viruses. Furthermore, synergistic combinations of NAC and existing antiviral drugs provide effective protect against lethal influenza viral infection in a mouse model have also been reported, further indicated that combination therapy target the host and virus factors is an attractive approach to prevention and therapy of influenza [52, 53, 56].

4.2 Glutathione

Glutathione (GSH), as a small molecule peptide with sulfhydryl, is an important antioxidant in many organisms. Acting as the most essential ROS scavenger[58], GSH can prevent injury of vital cellular components caused by free radicals, superoxide, lipid peroxides, and heavy metals [59]. This makes GSH can perform therapeutic effects on many diseases like chronic obstructive pulmonary disease (COPD). Study has suggested that viral replication and infectivity could be inhibited by GSH [60]. In the study of Cai, J et al. [61], GSH inhibited influenza virus-induced apoptosis and generation of virus particles notably. In addition, it also depressed viral matrix protein expression, virus-induced caspase activation and Fas upregulation. Moreover,

nutritional supplement formulation with GSH may antagonize the major pathogenic processes of H5N1 [62].

4.3 Other small-molecule antioxidants

There are still other small-molecule antioxidants that work on influenza viruses. A kind of isoprenoid phenol named bakuchiol produced an anti-influenza effect through influencing oxidative stress response of host cells [63]. Flavonoids baicalein and biochanin inhibited highly pathogenic avian H5N1 influenza A virus replication by reducing virus-induced ROS formation [64]. Polyphenols such as EGCG and quercetin showed anti-viral activity involving in multiple targets. For example, EGCG not only inhibited the replication of influenza virus, but also showed significant protective effects against oxidative stress through exhibiting antioxidant activity [65, 66]. Quercetin decreased alveolar macrophage superoxide production during influenza viral infection [67]. We also found quercetin showed direct anti-viral activity by inhibiting HA2 of influenza virus [68]. A recent research demonstrated that a biflavonoid isolated from Garcinia kola seeds, named Kolaviron possessed potent anti-influenza virus activity via its antioxidant and immunomodulatory effect [69].

5. Conclusion and prospective

Influenza virus invades the host cell, arising oxidative stress which mediates tissue damage, inflammation response and cell apoptosis. Accumulated studies have revealed that oxidative stress is often involved in multiple signaling pathways. Antioxidants represent a potential therapeutic option to fighting influenza. Hence, the importance of development novel compounds that block oxidative stress for research

and clinical use need to reinforce. Moreover, we speculate from the mentioned above that the combined application with antioxidants and anti-influenza drugs in current clinical use is likely to afford full protection to lethal influenza virus challenge. As a conclusion, based on the results we obtained from these studies and the literature data discussed above, increasing the awareness of the oxidative stress mechanism caused by influenza virus will not only offers new ideas for the pathogenesis of the virus, but also provides a therapeutic option for the prevention and the control of influenza virus infection, especially highly pathogenic human avian influenza.

Conflict of interest

The authors declared no conflict of interest.

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Figure captions

Figure 1 Schematic diagram of the role of oxidative stress in influenza virus infection. Oxidative stress occurs due to increase ROS production during influenza virus infection, while the virus-induced oxidative stress may contribute to several aspects of pathogenesis of influenza virus infection, including tissue damage, inflammatory response, and apoptosis. Antioxidants can block oxidative damage may developed a new therapeutic strategy to fight influenza virus infection.

Figure 2 The oxidative stress-activated signaling pathways. (A) Activation of the Nrf2-ARE signaling pathway. Inactive Nrf2 binds to Keap1, while oxidative stress can lead to dissociation of Nrf2 and Keap1, and the phosphorylated Nrf2 follows into the nucleus and bind to ARE and interact with transcription factors in the bZIP family. (B) Activation of thep38 MAKP signaling pathway. ASK is activated by oxidative stress, resulting in phosphorylation and activation of p38. The phosphorylated p38 translocates into the nucleus of host cells and is involved in the expression of cytokine genes. (C) Activation of the NF-κB signaling pathway. Under the conditions of oxidative stress evokes by influenza virus, IκBs is phosphorylate by IκB kinase, making IκB separates from P50/p65 heterodimer. Next, the activated NF-κB transfers into the nucleus and binds to specific DNA sequences, leading to follow-up reactions. Detail pathway information sees by BioCarta.

Table 1. Mechanisms of action of antioxidants

Drug(s)	Mechanism(s)	References
NAC	NAC inhibited H5N1 influenza virus replication and H5N1-induced production of pro-inflammatory molecules which was associated with oxidant sensitive pathways.	54
NAC	NAC alleviated the lung damage by inhibiting level of TLR4 protein.	55
NAC	Inhibitory activity of NAC against influenza A viruses appeared to be strain-dependent.	57
The combination of NAC with existing antiviral drugs	The combination of NAC with ribavirin or oseltamivir enhanced anti-influenza effects, indicating that these drugs have synergistic anti-viral effect via different mechanisms.	52;53
GSH	GSH inhibited viral replication and infectivity by regulating redox state in the host cells.	60
GSH	GSH blocked influenza viral infection *in vitro* and *in vivo* by inhibiting expression of viral matrix protein, caspase activation and Fas upregulation.	61
Bakuchiol	Bakuchiol enantiomer-selectively inhibited influenza A viral infection by activating Nrf2 pathway.	63
Baicalein and biochanin	Baicalein and biochanin inhibited highly pathogenic	64

	avian H5N1 influenza A virus replication by reducing virus-induced ROS formation.	
EGCG	EGCG exhibited inhibitory activities against influenza virus through significantly suppressing the increased ROS level.	65
EGCG	EGCG exerted anti-influenza A virus activity in MDCK cell culture by specific interaction with HA.	66
Quercetin	Quercetin decreased alveolar macrophage superoxide production during influenza viral infection.	67
Quercetin	Quercetin performed the inhibitory activity in the initial stage of influenza virus infection through targeting the HA2 subunit of influenza A virus hemagglutinin.	68
Kolaviron	Kolaviron protected mice from influenza virus infection involving in its antioxidant and immunomodulatory effect.	69

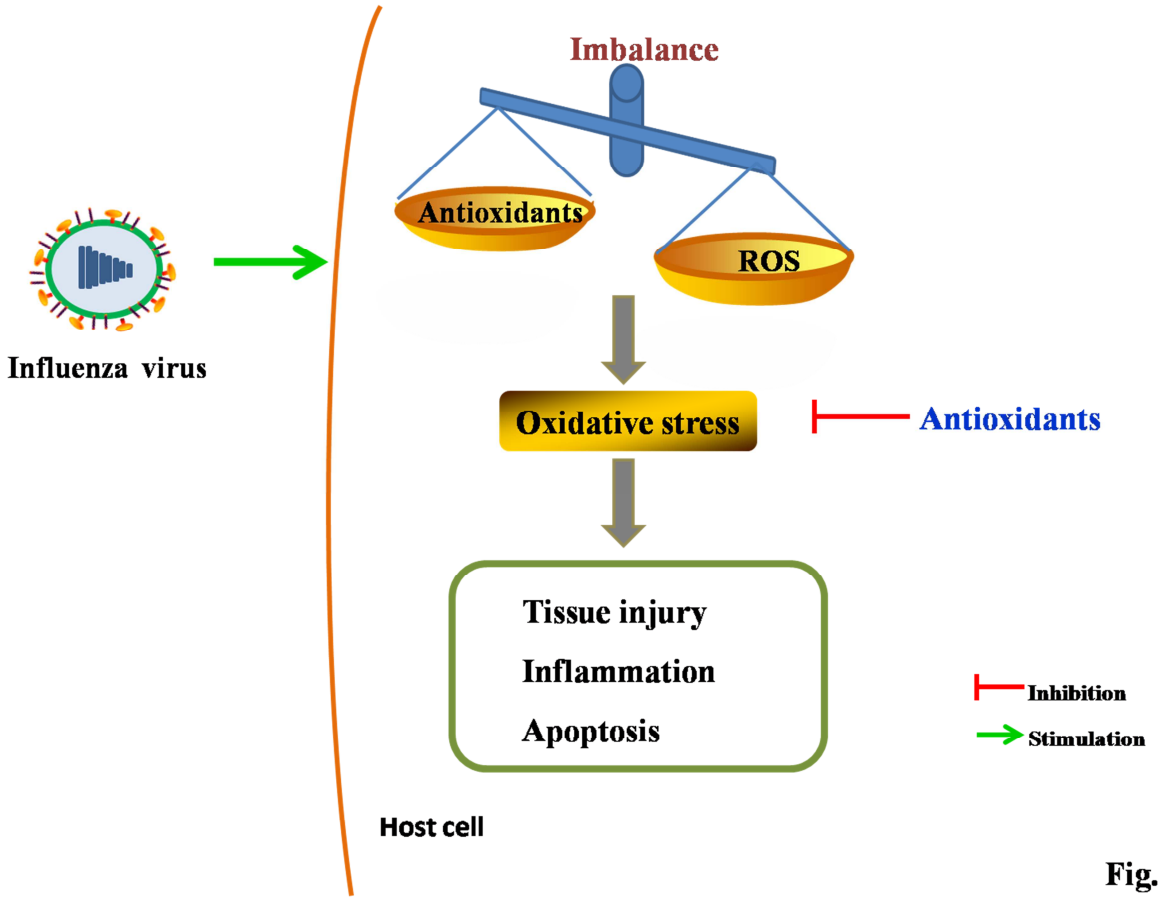

Fig. 1

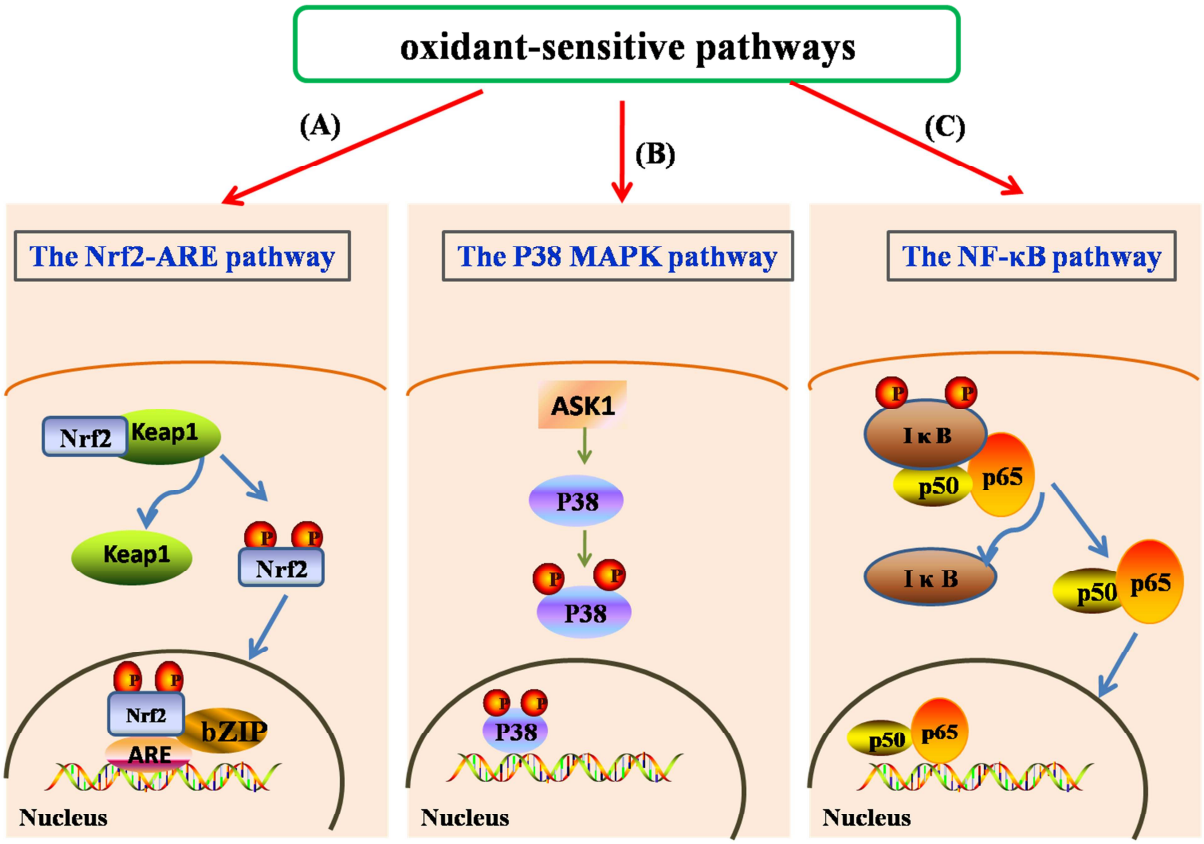

Fig. 2