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Host cellular signaling induced by influenza virus

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A wide range of host cellular signal transduction pathways can be stimulated by influenza virus infection. Some of these signal transduction pathways induce the host cell's innate immune response against influenza virus, while others are essential for efficient influenza virus replication. This review examines the cellular signaling induced by influenza virus infection in host cells, including host pattern recognition receptor (PRR)-related signaling, protein kinase C (PKC), Raf/MEK/ERK and phosphatidylinositol-3-kinase (PI3K)/Akt signaling, and the corresponding effects on the host cell and/or virus, such as recognition of virus by the host cell, viral absorption and entry, viral ribonucleoprotein (vRNP) export, translation control of cellular and viral proteins, and virus-induced cell apoptosis. Research into influenza virus-induced cell signaling promotes a clearer understanding of influenza virus-host interactions and assists in the identification of novel antiviral targets and antiviral strategies.

influenza virus, virus-host interaction, signal transduction

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Influenza, which causes thousands of deaths and huge economic losses every year, remains a significant public health problem worldwide. Influenza viruses exhibit high genetic variability. Rapidly generated virus variants can evade host acquired immunity against previously encountered strains. Large amounts of vaccines are produced to deal with seasonal influenza every year, but these vaccines cannot combat all influenza virus variants. It would be more problematic if there are newly emerging variants, such as the H1N1 variant that emerged in 2009. In influenza virus-infected cells, a series of signaling cascades can be stimulated at each stage of infection, some of which are important components of antiviral immunity, while others are essential for sufficient viral replication. Therefore, intracellular signaling cascades might be suitable targets for new antiviral strategies.

Generally, the signaling molecules in the host cell during viral infection can be classified into three groups: the receptors on the cell membrane, the adaptor molecules including kinases in the cytoplasm, and the transcription factors in the nucleus. During the infection of influenza virus, specific host receptors can recognize the viral proteins and/or genomic RNA and thus initiate a series of signal transductions. In this review we will discuss the main findings relating to the cellular signaling induced by influenza virus infection.

1 Recognition of viral components and innate immune signaling

1.1 Double-stranded RNA (dsRNA)-activated protein kinase (PKR) and downstream signaling

Protein kinase (PKR) which plays roles in the normal con-

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trol of cell growth and differentiation was first discovered for its ability to phosphorylate translation initiation factor eIF2 [1]. PKR can be induced by interferon (IFN) and activated by dsRNA, cytokines, growth factors and stress signals. PKR is an important sentinel kinase for viral double-stranded RNA (dsRNA) [2]. Influenza dsRNA activates PKR and triggers antiviral responses. A recent study indicated that the ribonucleoprotein of influenza B virus can also activate PKR and induce cellular antiviral responses [3]. At the onset of viral infection, activated PKR phosphorylates eukaryotic translation initiation factor 2 (eIF2), which inhibits cellular and viral protein synthesis [4,5]. Using PKR-deficient mice, Balachandran *et al*. [6] showed that PKR prevents viral replication by concomitantly assisting in the production of autocrine IFN. This finding indicated that PKR is an essential component of innate immunity that acts early in host defense, prior to the onset of IFN counteraction and the acquired immune response. PKR is also involved in influenza virus-induced apoptosis. Influenza virus infection induces augmented Fas expression; accordingly, transfecting the mutant PKR suppresses the augmented Fas expression and increases cell resistance to death upon virus infection [7]. The inducible overexpression of functional PKR in murine fibroblasts sensitizes cells to apoptosis induced by influenza virus and cells expressing a dominant-negative variant of PKR are completely resistant to influenza virus-induced apoptosis [8]. The influenza virus NS1 protein can directly bind to dsRNA or PKR to block the activation of PKR and evade the cellular antiviral effects induced by it [9,10].

1.2 Toll-like receptors (TLRs) and downstream signaling

Toll-like receptors (TLRs) are type I integral membrane glycoproteins and play crucial roles in early host defense against invading pathogens [11]. Currently, 11 members of the TLR family have been identified in mammals. TLR3/7/8, which are localized to the endosome membrane, are involved in viral pathogen recognition and the induction of type I IFNs [12]. TLR3 mediates dsRNA recognition and TLR7/8 mediates single-stranded RNA (ssRNA) recognition. The TLR family signaling cascades involve numerous downstream molecules, including MyD88, TAK1, TAB1, TAB2, TRAF6 and NF-κB [13,14]. In lung epithelial cells, the expression of TLR3 can be positively regulated by influenza A virus and dsRNA [15]. The TLR/TRIF pathway is essential for dsRNA and influenza A virus-induced NF-κB and IRF/ISRE activation [15]. Mitogen-activated protein kinase (MAPK) family members, especially Jun-N-terminal kinase (JNK) and p38 MAPK function importantly for antiviral immunity in influenza-infected cells. It has been shown that p38 MAPK and/or JNK are critical for the expression of several proinflammatory cytokines as well as apoptosis regulation [16–19]. Treatment with dsRNA and influenza A virus infection strongly upregulates the phosphorylation of three MAPK family members, JNK, p38, and extracellular signal regulated kinase (ERK)1/2, and enhances interleukin (IL)-8 as well as RANTES production [18]. In infected MDCK cells, the accumulation of viral RNA strongly activates JNK, which correlates with the induction of AP-1-dependent gene expression. Blockage of JNK signaling results in the reduction of AP-1 activity and the impairment of *IFN-β* gene expression [19]. Recent studies found that the MAPK-activated kinase RSK2 also plays a role in the cellular antiviral responses. The knockdown of RSK2 enhanced viral polymerase activity, the production of influenza virus, and reduced the activity of NF-κB and β IFN-dependent promoters [20]. p38 MAPK and/or JNK are showed to be activated by viral RNA, but whether and how TLRs interacts with them is still to be elucidated. Some *in vivo* experiments have shown that influenza virus infection upregulates TLR2 expression, but its function remains unclear [21,22]. TLR signaling can be negatively regulated by activating transcription factor-3 (ATF-3) [23]. When challenged with a sublethal dose of PR8 influenza virus, *ATF3*-knock-out mice were found to be delayed in body weight recovery and showed higher titers of the serum neutralizing antibody against PR8 five months postinfection compared with wild type mice.

1.3 Retinoic acid-inducible gene-I-like receptors (RLRs) and downstream signaling

The retinoic acid-inducible gene-I-like receptors (RLRs) are another major receptor system for detecting RNA viruses. RLRs consist of three members: RIG-I, MDA5 and LGP2. RIG-I and MDA5 are important cytoplasmic viral RNA sensors that play important roles in antiviral innate immunity. RIG-I and MDA5 recognize different viral RNAs. Along with the adaptor protein MAVS, they induce the activation of IRF-3/7 and NF-κB, which leads to the induction of IFNs and proinflammatory cytokines [24–26]. RIG-I is considered the central regulator of influenza A virus-induced expression of antiviral cytokines in human lung epithelial cells [27]. The overexpression of *RIG-I* gene constructs leads to a dramatic enhancement of IFN-β promoter-driven transcription in influenza A virus-infected epithelial cells. Dominant-negative *RIG-I* gene constructs inhibit influenza A virus-induced IFN-β promoter activity. IFN-α and Tumor Necrosis Factor (TNF)-α can enhance the expression of the components of RIG-I signaling pathways and enhance the expression of antiviral cytokines [28,29].

1.4 Non-structural protein and the innate immune response

Influenza infection induces *IFN* gene expression and other innate immune responses, but influenza viruses have also evolved numerous strategies for evading host immunity.

Non-structural proteins play important roles in these processes.

Non-structural protein 1 (NS1), a viral antagonist of IFN expression and its downstream effects, is the most important non-structural protein of influenza virus [30]. NS1 binds to dsRNA and sequesters dsRNA from recognition by pattern recognition receptors (PRRs) [9,10,31]. Furthermore, NS1 interferes with viral ssRNA that contains free 5′ triphosphate groups, a pattern recognized by PRRs [32]. It was also reported that NS1 affects viral RNA recognition by directly binding to RIG-I and suppresses IFN-β production consequently [33–35]. NS1 can also specifically block TRIM25-mediated RIG-I ubiquitination and repress RIG-I signaling [36]. In addition, NS1 can interact with the molecules involved in the transcription and translation of type I IFNs, repressing type I IFN production [37].

The PB1-F2 protein, another non-structural protein of influenza virus, has several functions in influenza virus infection. PB1-F2 can affect influenza virus-induced apoptosis. PB1-F2 localizes at the inner and outer mitochondrial membranes and mediates higher susceptibility to influenza virus-induced apoptosis [38]. PKC-mediated PB1-F2 phosphorylation enhances the induction of apoptosis in monocytes [39], which may be caused by the interaction of PB1-F2 with the mitochondrial membrane proteins ANT3 and VDAC1. This interaction leads to cytochrome C release via holes in the mitochondrial membrane [40]. PB1-F2 is important for the pathogenicity of influenza virus, as indicated by the results of previously reported animal experiments [41,42]. PB1-F2 can upregulate the expression of cytokines in infected lungs. This upregulation may be induced by the interaction between PB1 and PB1-F2, which leads to high viral polymerase activity and viral RNA accumulation in the infected cells [43].

2 Protein kinase C (PKC) and influenza virus entry

Protein kinase C (PKC) is part of a large family of serine/threonine kinases activated by many extracellular signals. PKCs are involved in a multitude of physiological processes [44]. The close relationship between PKC and sodium ion transport, important for maintaining the low pH in the endosome, has been confirmed by the findings of numerous studies [45–48]. PKC has also been shown to be critical for the entry of enveloped viruses (receptor-medicated endocytosis) [49]. Upon influenza virus infection, the hemagglutinin rapidly activates PKC [46,50] and it has been shown that a specific inhibitor of PKC prevents influenza virus replication by inhibiting the entry of the virus [51]. Similar results have also been reported in cells expressing a phosphorylation-deficient form of PKC [52]. Taken together, these studies demonstrate that PKC plays an important role in influenza virus entry.

3 The Raf/MEK/ERK pathway and ribonucleoprotein (RNP) export

The Raf/MEK/ERK signal transduction cascade belongs to the family of MAPK cascades. Raf/MEK/ERK signaling leads to stimulus-specific changes in gene expression, alterations in cell metabolism, or the induction of programmed cell death. Thus, Raf/MEK/ERK signaling controls cell differentiation and proliferation [53]. Ribonucleoprotein (RNP) formation and nuclear export are important steps in the life cycle of influenza virus. Influenza virus matrix protein and temperature both play important roles in these processes [54,55]. Among the different signalings activated by influenza virus, Raf/MEK/ERK cascade is required for an efficient nuclear RNP export as indicated by several studies [56,57]. Inhibition of Raf signaling results in nuclear retention of viral RNP and the concomitant inhibition of virus production [57]. Influenza virus hemagglutinin (HA) membrane accumulation and its tight association with lipid-raft domain trigger the activation of MAPK cascades via PKC-α activation and RNP export [58]. HA membrane accumulation is enhanced by the higher polymerase activity of influenza virus, resulting in upregulation of the MAPK cascade and more efficient nuclear RNP-export, along with virus production [59].

4 Eukaryotic translation initiation factor 4e (eIF4E) and viral protein translation

Influenza virus can efficiently shut off host cell protein synthesis and selectively translate viral RNA. The synthesis of viral mRNA is primed by short capped oligonucleotides which are scavenged from host pre-mRNAs [60,61]. De-capped host mRNA would probably be more susceptible to degradation. The NS1 protein of influenza virus can also inhibit the nuclear export of host mRNA and the splicing of pre-mRNA [62,63]. Sequences within the 5′ untranslated regions (UTRs) play a critical role in the selective translation of influenza viral mRNA [64]. The NS1 protein functions to increase the initiation rate of translation of viral mRNAs by interacting with the 5′-terminal conserved sequences of viral mRNAs [65–68]. Eukaryotic translation initiation factor (eIF) 4G Ⅰ, a subunit of cap-binding complex eIF4F, and poly(A) binding protein 1 (PABP1) are identified to be cellular targets of NS1 that support the role of NS1 in protein translation [69,70]. By binding to eIF4G Ⅰ, NS1 recruits eIF4F to the 5′ UTR of viral mRNA and activates translation of viral mRNA. eIF4E which is another subunit of eIF4F is dephosphorated during influenza infection. eIF4E dephosphorylation which may lead to a decrease of its cap-binding activity may contribute to the influenza viruses-induced inhibition of the cellular mRNA translation [71]. In virus-infected cells, activation of PKR, an inter-

feron-induced kinase, may induce global inhibition of translation initiation through phosphorylation of the eIF2α. To maintain a certain level of cellular protein translation, influenza viruses utilize at least two strategies, involving cellular $p58^{IPK}$ (Protein Kinase Inhibitor p58) and viral protein NS1, to block PKR activity [72,73].

Influenza viruses carry out temporal regulation of their own viral gene expression [74,75]. Nucleoprotein (NP) and NS1 protein are highly expressed at early times after infection, while HA, neuraminidase (NA) and matrix protein (M1) are expressed mainly at late times after infection. It is proposed that the translational efficiency of viral mRNA is subjected to regulation and the 5′ UTR of influenza virus mRNAs may be important for this translation control. However, the exact mechanisms have not been identified [76].

5 Phosphatidylinositol-3-kinase (PI3K)/Akt signaling pathway and viral infection

The activation of phosphatidylinositol-3-kinase (PI3K)/Akt signaling is a hallmark of cell survival [77,78], and plays important roles in a wide range of physiological processes. Recent studies found that PI3K/Akt signaling is a strategy employed by viruses to slow down apoptosis and prolong viral replication during acute and persistent infections [79]. In influenza A virus-infected cells, activation of PI3K is mediated by the viral NS1 protein, which binds directly to the p85β regulatory subunit and causes the PI3K-dependent phosphorylation of Akt [80–84]. Akt directly phosphorylates caspase 9, thereby inhibiting the activation of this apoptotic protease. Glycogen synthase kinase (GSK)-3β, another apoptosis modulator, is also phosphorylated and inactivated by Akt. Consequently, viral-induced apoptosis is suppressed by activated Akt signaling [83,85]. Akt signaling also protects influenza-infected cells from fast apoptosis through the p53-dependent pathway. In influenza-infected CV-1 and MDCK cells, nuclear accumulation and phosphorylation of p53 show low constitutive levels at early time of infection, whereas both are markedly elevated at the late infection stage (17–20 h postinfection) [86]. The PI3K/Akt pathway was recently reported to negatively regulate the JNK pathway via ASK1, thereby inhibiting JNK-dependent, Bax-mediated apoptosis during influenza A virus infection [87].

Besides promoting efficient viral replication by suppressing apoptotic signaling, the PI3K/Akt pathway has other important roles in influenza A virus propagation [79,88]. Before influenza A virus NS1 protein was found to activate PI3K/Akt signaling, PI3K was reportedly activated in response to dsRNA and mediated the activation of transcription factor IFR-3. Blocking PI3K signaling has been shown to impair the dimerization of IRF-3 and reduce IRF-3-dependent promoter activity. Furthermore, PI3K regulates an early step of viral entry [89]. In A549 cells, it was reported that the PI3K/Akt signaling pathway is activated independently of viral attachment and entry, and specific inhibitors of the PI3K/Akt pathway significantly suppress viral RNA synthesis and protein expression [90]. PI3K/Akt signaling includes many downstream effector molecules associated with cell survival, proliferation, differentiation, morphology and apoptosis. The suppressed viral propagation caused by blocking PI3K/Akt signaling may be a composite effect, and further studies focusing on the downstream processes of the PI3K pathway are necessary.

6 The complexity of the influenza virus–host interaction

Influenza virus-induced signaling involves a complex network of signaling and effector molecules (Figure 1). Some signaling pathways play multiple roles in viral infection. NF-κB, generally considered a key transcription factor in the production of type-I IFN and other antiviral cytokines [6,15,91–95], has been identified as a prerequisite for influenza virus infection [96]. NF-κB signaling can regulate influenza virus RNA synthesis. Knocking-down of p65 NF-κB molecule reduces influenza virus replication and viral RNA synthesis [97]. In addition, the NF-κB-dependent induction of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and Fas/FasL is crucial for efficient influenza virus propagation [98]. Similarly, the viral RNA recognition receptor RIG-I, important for the production of antiviral cytokines [28,99–101], is also involved in the SOCS1- and SOCS3-mediated negative regulation of the innate immune response [102]. The RIG-I/MAVS signaling pathway activates caspase-3, suggesting a role for this receptor beyond the innate cytokine response [103].

7 Conclusion

Influenza viruses afflict millions of people each year and cause serious medical complications. Because of the high genetic variability of influenza viruses, the development of effective vaccines against pandemic influenza is still an ongoing challenge. Some mutant influenza viruses are resistant to conventional antiviral reagents. In recent years, the highly pathogenic avian influenza A virus H5N1 and influenza A virus H1N1 have presented new threats. In infected cells, viral-induced signaling pathways are required for effective antiviral responses or for sufficient viral replication. Receptor-mediated viral RNA recognition by the host plays a central role in antiviral signaling responses. It activates a series of kinases and important transcription factors, as well as induces the expression of antiviral genes,

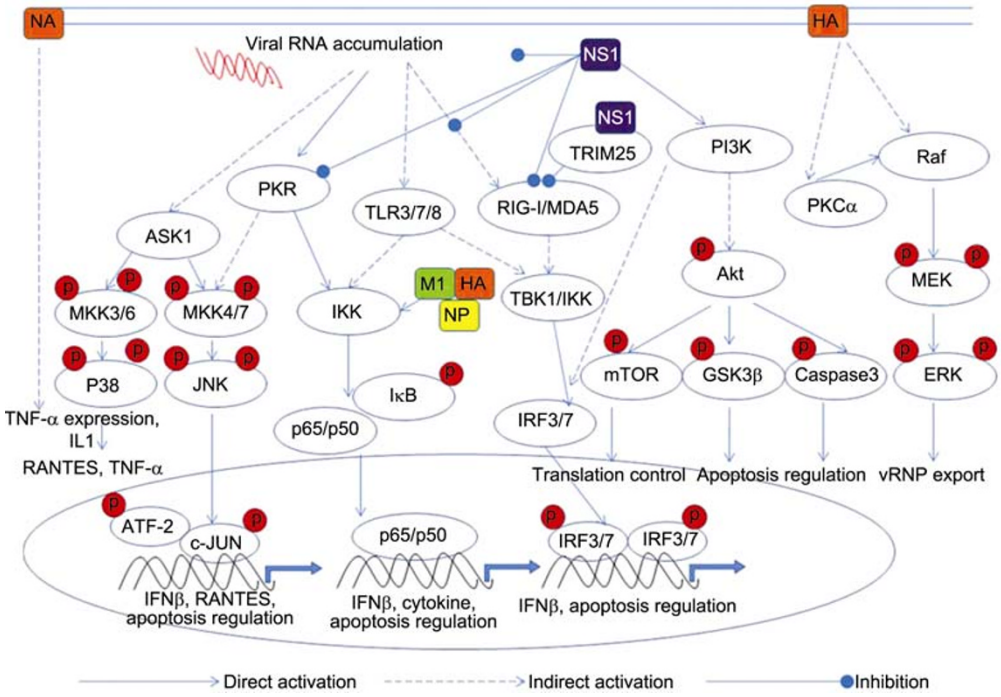

Figure 1 Host cellular signaling induced by influenza virus. Akt, protein kinase B (PKB); ATF, activating transcription factor; ASK, apoptosis signal-regulating kinase; IκB, inhibitor of κB; IRF, interferon regulatory factor; MDA5, melanoma differentiation associated gene 5; MKK, mitogen-activated kinase kinase; mTOR, mammalian target of rapamycin; Raf, rapid accelerating fibrosarcoma; TBK, TANK-binding kinase; TRIM, tripartite motif.

including IFNs and other cytokines. Besides viral RNA, some viral proteins can also induce the innate immune response through antiviral signaling pathways. On the other hand, influenza virus takes advantage of cell signaling for its propagation at almost all stages of its life cycle. For example, activated PKC is required for viral entry. Raf/MEK/ERK signaling is essential for viral RNP export. The NS1 protein activates several signaling pathways to ensure efficient influenza virus replication, including the suppression of the innate immune response, the enhancement of viral RNA synthesis, increased viral protein expression and regulation of host cell apoptosis.

A persistent problem in viral-induced signaling research is the widespread use of tumor cell lines. Their changed physiological and biochemical properties may cause discrepancies between *in vitro* studies and *in vivo* studies. Influenza virus-induced signaling involves a complex network of different overlapping molecules and signal cascades. Research on a single molecule or pathway cannot, therefore, provide a comprehensive description of virus–host interactions. Combining genome, transcriptome and proteome research methods may prove helpful in obtaining a clearer understanding of virus–host interactions. Recently, two research groups have used large-scale RNAi screening to analyze these interactions, and got some important new discoveries [104,105]. Greater insight into viral-induced signaling will not only increase our understanding of the interaction between influenza viruses and host cells, but will also potentially provide novel antiviral therapeutic targets.

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