Review

Activation of c-Jun N-terminal Kinases by Ribotoxic Stresses

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The c-Jun N-terminal kinases (JNKs) are classic stress-activated protein kinases. Many cellular stresses have been shown to stimulate JNK activation. In this review, we focus on ribotoxic stresses based on their multiple biological potencies including anti-HIV-1 activity. Some of the functions of ribotoxins and the signaling transduction pathway that mediated are mentioned. Different from other stimulators, ribotoxic stresses act on special motifs of 28S rRNA in translationally active mammal ribosomes. Binding and damaging on the motif leads to JNK activation and subsequently biological response to the signal initiator, which is named ribotoxic stress response. *Cellular & Molecular Immunology*. 2005;2(6):419-425.

Key Words: ribotoxic stress response, JNK, MAPK, apoptosis, ribotoxin, ribosome inactivating protein, HIV

Introduction

Cell signaling transduction is a cascade of amplification process when cells are stimulated and response to the stimulators. A series of protein kinases are activated and, in most cases, eventually act on nuclear transcription factors involved in gene expression. The most ancient and evolutionarily conserved signaling pathway is that mediated through mitogen-activated protein kinases (MAPKs).

There are three main families of MAPKs in mammalian species: the extracellular signal-regulated protein kinases (ERKs), the p38 MAP kinases and the c-Jun N-terminal kinases (JNKs). JNKs are classic stress-activated protein kinases. Three highly related but distinct gene products, JNK1, JNK2 and JNK3, belong to this family, each possessing several isoforms as a result of variable mRNA splicing. JNK1 and JNK2 show a broad tissue distribution, each possessing 4 isoforms with common molecular weights of 46 kD and 55 kD, whereas JNK3 is expressed predominantly in neurons but also in cardiac smooth muscle and testes, which has 2 isoforms with molecular weights of 48 kD and 57 kD (1, 2).

JNKs are known as the only kinases to activate transcription factor c-Jun by phosphorylating at its N-terminal region. They are activated through a sequential signaling cascade. The upstream kinases of JNKs are the dual specificity MAPK kinases (MAPKKs) MKK4 and MKK7. When cells are exposed to stresses, MKK4 or MKK7 is phosphorylated and thus activated by multiple upstream MAPKK kinases (MAPKKK) including members of the MEKK group (MEKK1 through 4), the mixed-lineage protein kinase group (MLK1, MLK2, MLK3, DLK and LZK), the ASK group (ASK1 and ASK2), TAK1 and TPL2 (2). A downstream kinase only responses to a certain group of its upstream kinases, which endows partial specificity to the signaling transduction pathway (3). Both MKK4 and MKK7 can be phosphorylated by MLK1-3 while DLK acts on the JNK pathway only through MKK7 (4, 5).

The JNK signal transduction pathway is preferentially activated in response to environmental stresses and by the engagement of cell surface receptors (6). Targets of JNKs are mostly transcription factors, including c-Jun, activating transcription factor ATF-2 (7), and ETS-containing factors such as Elk1 (8). Other targets regulated by JNK-mediated phosphorylation include insulin receptor substrate 1 (IRS-1) (9) and Bcl-2 (10).

Several drugs have been reported to inhibit JNKs activation. CEP-11004 is an indolocarbazole analogue reported by Cephalon Inc., which could be used as a JNK pathway inhibitor (11) by inhibiting MLK1-3 (5). Another JNK inhibitor, SP600125 (anthra [1,9 cd] pyrazol-6(2H)-one 1,9 pyrazoloanthrone), inhibited JNK1, 2, and 3 isoforms with similar potency but exhibited greater than 300-fold selectivity against related MAP kinases ERK1 and p38-2, and the serine threonine kinase PKA (12). P38 inhibitor SB202190 also inhibited JNK activity at high concentration

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(50 μM) (13) while could inhibit p38 kinases at a much lower concentration (< 10 μM) (14). Other JNK inhibitors include JIP-1 (15), CEP-1347 (16), and an engineering peptide inhibitor which was obtained by linking the minimal 20-amino acid inhibitory domain of the islet-brain (IB) protein to the 10-amino acid sequence of HIV-Tat, and thus was allowed rapid translocation through the cell membrane (17). Interestingly, 1α,25-dihydroxyvitamin D_3 had been shown to be a good inhibitor for JNKs that were activated by heat shock, TNF-α, sorbitol, H_2O_2 or ultraviolet and thus could protect keratinocytes from ultraviolet (UV)- and chemotherapy- induced damage (18).

The consequence of JNK activation is dependent on stress species and the target cell types. C-Jun, a main component of the AP-1 transcription factor(s), was implicated in the regulation of a wide range of biological processes including development, differentiation, transformation and apoptosis (2, 19, 20).

Studies using strategies to either overexpress or underexpress c-Jun protein or block its phosphorylation have provided evidence that c-Jun was an important mediator of apoptosis induced by various cytotoxic agents including some chemotherapeutic drugs (21), alkylating agents (22), ultraviolet-C (UVC) and γ-radiation (23). In murine fibroblasts, the absence of JNKs caused the failure to release cytochrome c (24). But more evidence showed that JNK activation can increase cell survival. Inhibition of c-Jun activity in neuronal cells was found to protect these cells from nerve growth factor withdraw (25). Convincing evidence was from human tumor cell lines with site-directed mutation on *c-jun* gene (S63A, S73A), expressing high level of unphosphorylatable c-Jun, which was resistant to cisplatin-induced cytotoxicity (26). And similar evidence was found in NIH3T3 cells from c-Jun knock-out mice exposed to the same reagent, showing that activation of JNK was necessary for cisplatin-induced caspase activation and apoptosis (21). All of these findings suggested that N-terminal phosphorylation-dependent activation of c-Jun plays an important role in protecting human tumor cells against DNA damage-induced apoptosis.

Ribotoxic stresses and JNK activation

Cellular stresses activating JNKs

Like other MAPKs, JNK family members have a typical activation motif of Thr-Pro-Tyr (TPY). They are activated by upstream kinases MKK4 and MKK7 after being phosphorylated at both threonine and tyrosine in this motif (3). Many cellular stresses have been shown to stimulate JNK activation. These stresses include exposure to UV irradiation (27), heat and osmotic shock (20, 28), oxidative stresses (6), inflammatory cytokines (6), growth factor withdraw (29) and DNA damage agents, such as cisplatin (21), etoposide, methyl methanesulfonate (MMS), doxorubicin, daunorubicin, mitoxantrone, etc. (21, 26). Among these stresses, we are more interested in ribotoxic stresses, because many ribosome inactivating proteins (RIPs) have been demonstrated to possess different biological activities including inhibition of HIV-1 replication.

Activation of JNKs is specifically mediated by certain stimulators. Several different but coexisting mechanisms guarantee the specificity of JNK signal transduction (30, 31): (i) modulation by scaffold proteins. Scaffold proteins are organizers and facilitators of the MAPK signaling pathway. In mammalian cells, for example, scaffold protein JIP1 forms a signaling cassette together with JNK1, JNK2 and their upstream kinases MKK7 and MAPKKK MLK1; (ii) sequential physical interaction in cascade response. JNK1/2 binds to the N-terminal of MKK4 and synchronously interacts with the catalytic motif of MEKK1. Once downstream kinases are activated, such a sequential interaction is dissolved. Modulation by scaffold proteins and sequential physical interaction can act at the same time. Both mechanisms contribute to the specificity of signaling transduction, but the latter one also greatly amplifies the original signal. (iii) ligands and inhibitors for cell-surface receptors that feed back into MAPK cascades. This auto-secretory loop of positive and negative feedback may form a special space manner of MAPK activation. (iv) special distribution of the components of JNK transduction pathway. Some of the signal molecules are coexistent with cell skeleton. Phosphorylation of these components also influences cell movements.

Ribotoxic stress response

Ribotoxic stress response was well defined by Iordanov et al (32). The typical type I RIPs ricin A and α-sarcin were found to activate JNK1 and its activator SEK1/MKK4 by sequence-specific rRNA damage to the α-sarcin/ricin loop (which is fairly conserved from prokaryocytes to eukaryocytes) in the 28S rRNA of rat liver cells and could induce the expression of immediate-early genes *c-fos* and *c-jun*.

Other RIPs, including mistletoe lectin II (33), trichothecene mycotoxins (7), shiga toxin (34), onnamide A and theopederin B (35) could also activate JNK(s) as ricin A and α-sarcin did. Recently, we found that trichosanthin (TCS) can also activate JNKs in C8166 cells in a time- and dose-dependent manner (data not published).

In addition to RIPs, antibiotic anisomycin, UVB and UVC also activated JNKs in a ribotoxic stress response manner (36, 37). Unlike genotoxic, hyperosmotic and pro-inflammatory stressors, ribotoxic stress response requires the presence of translationally activated ribosomes at the time contacting with the drug, suggesting that the stress response (JNK activation) is originated from damage to the active ribosomes (32, 36, 37). And ribotoxic stresses, such as UVB (37) and trichothecene deoxynivalenol (DON) (38) potentiated JNK activity rapidly (usually within 5 minutes), as compared to oxidative stresses that did in a slow kinetics (37).

It should be noted that protein synthesis inhibitors damaging ribosomes do not necessarily activate JNKs in cells. Inhibition of protein synthesis *per se* does not potentiate JNKs. Antibiotics anisomycin, pactamycin and emetine are

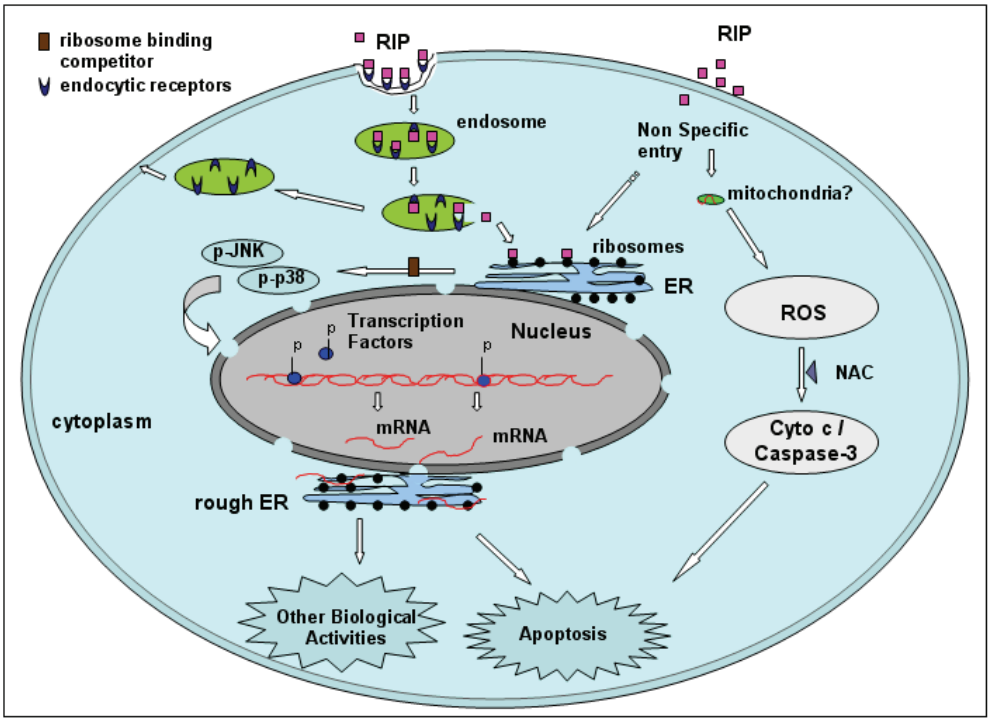

Figure 1. Ribotoxic stress response. Induced cell apoptosis was shown. The figure is partly referred to reference 53.

protein synthesis inhibitors targeting at translation elongation. At the concentration inhibiting 95% of protein synthesis, anisomycin strongly activated JNK(s) while pactamycin and emetine completely failed to (32).

Mechanism of ribotoxic stress response

Efforts are being made on the mechanism of ribotoxic stress response, though the entire pathway of signal transduction is still unclear. Some of the upstream kinases identified are double-stranded RNA-activated protein kinase R (PKR) (38) and hematopoeitic cell kinase (Hck/Src) (7) activated by DON which leads to cell apoptosis. Cellular response to UV exposure had been considered possibly to be the consequence of UV-induced damage to DNA for a long time. But Devary et al found that enucleated HeLa cells were still able to activate JNK1 and NF-κB after irradiation (39). This was powerful evidence supporting that all of the JNK1 activation components of the UV-induced signal transduction pathway including the stress-sensing molecules must exist outside of the nuclear membrane (39).

In the case of RIPs, it seems that activation of JNK(s) is originated from ribosome damage (Figure 1). As mentioned above, inhibition of protein synthesis and activation of JNK(s) can be dissociated by pactamycin or emetine pretreatment before exposure to ribotoxic stresses (32). The ability of certain members of trichothecene family to inhibit protein synthesis and activate JNK/p38 kinases was dissociable. Those who inhibited protein synthesis without activating JNK/p38 kinases inhibited the function (i.e. activation of JNK/p38 kinases and induction of apoptosis) of apoptotic trichothecenes and anisomycin. The ribosome binding competitor harringtonine, a protein synthesis inhibitor, could abolish the activation of JNK/p38 kinases as well as induction of apoptosis by trichothecenes and anisomycin (40).

Damage in the α-sarcin/ricin loop which inhibited protein synthesis requires the translocation of ribosome inactivating proteins into cytoplasm through endocytosis and released from the vesicles without enzymatic degeneration before they anchor at ribosome rRNA site-directedly. TCS, for example, entered cells through two different pathways, which was dependent on cell types. One took a slow and nonspecific route, as did in H35 cells. The other was mediated by two endocytic receptors LDL receptor-related protein (LRP) and megalin as observed in JAR cells and proximal tubule epithelial cells respectively. TCS entered JAR cells in this manner with a swift transportation across the membrane and rapid accumulation of intracellular TCS. In order to trace the intracellular distribution of invaded TCS, the protein was conjugated with gold particle (Au) and observed under a transmission electron microscope. Most of TCS was found to bind to coated pits on the JAR cell surface and be rapidly internalized within an hour (41, 42). Others found that TCS preferred to bind to negatively charged phospholipids-containing membranes and such binding was dependent on the pH value and the ionic strength (43). This might account for how TCS leaked from endosomes. Though previous work suggested that JNK activation triggered by ribotoxic stresses

originated from ribosome damage, it still can not exclude the possibility that receptors on cell membrane play an important role in the signal transduction pathway.

Functions of ribotoxins

Inhibition of protein synthesis

Ribotoxins are characterized by their site-directed action on ribosomes, thus leading to inhibition of protein synthesis. The most studied ribotoxins that inhibit protein synthesis are RIPs.

RIPs are a group of cytotoxic proteins acting on eukaryotic ribosomes (44). Most RIPs are from higher plants except α-sarcin from fungi, Shiga toxin and Shiga-like toxin from bacteria. Some of these ribotoxins (ricin A and TCS) inactivated 60S ribosomal subunits by hydrolyzing a single N-C glycosidic bond of the adenosine residue at position 4324 (A_{4324}) in 28S rRNA of mammalian cells adjacent to the α-sarcin cleavage site (G_{4325}-A_{4326}) (45, 46). Others provided evidence that Shiga toxin, Shiga-like toxin II variant and ricin specifically removed A_{3732}, located at 378 nucleotide from the 3' end of 28S rRNA when microinjected into Xenopus Oocytes (47).

Induction of cell apoptosis

TCS is an abortificient purified from root tubers of the Chinese medical herb *Trichosanthes kirilowii* Maxim, with a molecular weight of 27 kD. Based on structural and functional properties, the protein belongs to the family of single-chain RIPs, which inhibits protein translation in cell free systems (48, 49). Studies from our laboratory and others' showed that TCS could induce cell apoptosis in H9 cells (50) and JAR cells (51).

Other ribotoxins that can induce cell apoptosis include onnamide A and theopederin B (35), DON (7), Shiga toxin 1 (34), etc. Cell apoptosis induced by these drugs may involve two different mechanisms (Figure 1). One is the consequence of ribotoxic stress response, which requires JNK activation as mentioned above. Ribosome binding competitor (emetine, etc.) can rescue cells from doom while N-acetyl cysteine (NAC, a scavenger of reactive oxygen intermediates) can not. Blocking JNK and p38 kinase activation with inhibitor SB202190 was reported to prevent cells from Shiga toxin 1 triggered death (34). The other mechanism might function through oxidative stress response, as suggested by Zhang et al. (51). Anti-oxidant reagents such as NAC and Trolox but emetine abolished reactive oxygen species (ROS) induced apoptosis (37).

Interestingly, ribotoxin-induced cell apoptosis is cell-type dependent. The best known ribotoxic stress response inducer anisomycin induced rapid apoptosis in human lymphoid cells (52) but did so weakly in HeLa cells (35). This might be due to binding of anisomycin to the different site on ribosomes and thus generating a different mode of the signal that activated downstream kinases. Transient activation of p38 kinases and JNKs by anisomycin seemed insufficient to induce apoptosis in HeLa cells.

Selective cytotoxicity of ribotoxins may have protential clinical significance. TCS exhibits many biological and pharmacological properties, including antitumoral, immuno-suppressive and antiviral activities (53). This traditional Chinese medicine was and is still used to induce mid-term abortion and to treat choriocarcinoma in China. Our laboratory found that TCS selectively killed leukemia-lymphoma cells (54).

Anti-HIV activity of RIPs

TCS is also the first RIP found to inhibit human immunodeficiency virus type 1 (HIV-1) replication in both acutely infected T lymphoblastoid cells and in chronically infected macrophages *in vitro* (55, 56). Phase I/II clinical trials with TCS alone or in combination with zidovudine or dideoxyinosine showed that TCS decreased the serum HIV-1 p24 antigen level and increased the $CD4^+$ T cell number in HIV-1 infected patients (56, 57). In addition to TCS, many other RIPs, including MAP30, GAP31, DAP30, DAP32, TAP29, PAP, Bryodin, alpha-momorcharin (MMC) and trichobitacin, have been reported to inhibit HIV-1 replication *in vitro* (58-64).

The exact anti-HIV-1 mechanism of TCS is far from clear. It is generally believed that its ribosome inactivating activity contributes to this action, with the *N*-glycosidase activity that depurinates adenine 4324 of 28S rRNA. However, other mechanisms may also exist, including TCS-induced ribotoxic stress response. Not all RIPs have anti-viral activities (65). Two TCS mutants, TCS_{C19aa} and TCS_{KDEL} having 19 amino acids more and a KDEL signal sequence added to the C-terminal sequence, retained all RI activity but subsequently lost most of the anti-HIV-1 activity (66).

Prospects

Early studies demonstrated that MAPK signal transduction plays an important role in HIV replication. The proinflammatory cytokines interleukin (IL)-1 and tumor necrosis factor (TNF-α), which activated p38 kinases, promoted HIV type 1 viral replication in U1 and peripheral blood monocytes. In the presence of a specific p38 MAPK inhibitor (SB203580), HIV production induced by the cytokines and stress responses was greatly suppressed (67).

MAPK signal transduction also plays an important role in AIDS pathogenesis. *In vivo* infection of lymphatic tissues by HIV-1 leads to enhanced apoptosis. Most of the killed cells were uninfected bystander cells instead of infected ones. In infected cells, HIV-1 Nef associated with apoptosis signal-regulating kinase 1 (ASK1), an upstream kinase of JNK pathway, and thus inhibited the Fas and TNF-α death-signal pathway through which the activated bystander cells were killed (68). This might partly account for why HIV-1 particles mainly killed bystander cells while protected infected cells.

Although we still need time to learn the exact role of MAPK activation in HIV-1 replication and pathogenesis, it is definitely sure that several HIV-1 particle proteins such as

Nef (69), Tat (70), gp120 (71) can activate JNKs *in vitro* and consequently stimulate HIV-1 transcription (69) or induce self-perpetuating permissivity for productive HIV-1 infection (72). HIV replication is strictly and accurately regulated by signal transduction in host cells. Any drug that disrupts this signal transduction chain could more or less influence the viral particle replication and pathogenesis. The more we learn about what have happened during viral particle replication, the closer we are to the destination of controlling the disease.

Ribotoxins are still alluring approaches for researchers to investigate due to their effective anti-tumoral, anti-viral, anti-fungal, insecticidal and many other activities. TCS is a well-known anti-HIV-1 drug. But the severe antigenicity of this compound stunted its further clinical use in anti-HIV-1 area (53) and the half-life of this protein in human body is too short. Researchers hope to obtain a mutant by engineering structural reform of this compound which retains most of the anti-viral or anti-tumoral activity but abandons the above shortcomings. Unfortunately, no pleasing mutant has been gained till now. PEG-conjugated TCS, though lasting its half-life in pharmacokinetics, loses much anti-HIV-1 activity correspondingly (73). The unclear mechanism of the function of this ribotoxin is an obstacle for structural reform. More efforts should be focused on the cell signaling pathway induced by anti-viral candidates.

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