Introduction

The influenza A virus subtype H5N1 is highly pathogenic virus that becomes endemic in Asia, the Middle East, Europe and Africa (Al-Azemi A et al., 2008). As of 2010, WHO has reported there were 512 confirmed human cases of H5N1 worldwide of which 304 were fatal. However, not all infected individuals died from severe respiratory illness. This is because the virus not only attacks respiratory system, but also attacks other vital organs including the brain, where it can induce severe and deliberating encephalopathy or acute encephalitis. In mice, the virus was reported to cause encephalitis without prior host adaptation and virus antigens were detected in nerve and glial cells (Jang et al., 2009). Similar findings were also reported in feline including tiger. H5N1 virus evolved from the Hong Kong strain caused the second outbreaks in Asia resulting in acute encephalitis leading to a death of two Vietnamese siblings who initially developed a severe diarrhea without apparent respiratory dysfunction followed by rapid progressive coma. Moreover, the virus was isolated from their serum and cerebrospinal fluid specimens (de Jong et al., 2005). This might be similar to the notorious Spanish flu (influenza A virus subtype H1N1) that caused the great 1918 pandemic and gave rise to global outbreak of encephalitis and its neurological sequelae, including post-encephalitic parkinsonism.

The presence of infectious virus particles and viral genomes in the brain and elevated levels of pro-inflammatory cytokines in the cerebrospinal fluid of patients who died from H5N1 encephalitis strongly indicate that brain cells actively interact with H5N1 viruses during the course of infection. Human infection of H5N1 could impair normal brain functions by causing cell death and subsequently production and spreading of infectious virus progeny within the nervous system. Generally, acute inflammation is beneficial to infected host in limiting the survival and proliferation of invading pathogens and promotes tissue regeneration. Unfortunately, prolonged and excessive

inflammation within the brain is highly detrimental leading to the exacerbation of neurodegenerative process. This is due to the fact that nerve cells compared to other types of brain cells are the most susceptible cells to inflammatory reaction during the course of virus infection, in addition of being the prime target of the virus (Bissel et al., 2012) .

In the current study, we investigated neuroinfectious capacity of H5N1 (A/Thailand/ NK165/2005) isolated from plasma of infected individual during the third wave of Thailand outbreaks using human neuroblastoma SH-SY5Y cells. This is due to lack of information regarding neuroinfectivity of this variant. Moreover, using this isolate we aimed to identify host cell proteins that might be involved in H5N1 entry to nerve cells using one dimension virus overlay protein binding assay (1D-VOPBA) coupled with LC-MS/MS analysis.

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Survey of related literatures

Avian influenza is caused by H5N1 influenza A virus, that belongs to family Orthomyxoviridae. H5N1 has become endemic in Asia, the Middle East, Europe and Africa and has been recognized as a highly pathogenic virus (Al-Azemi A et al., 2008). The virus can be directly transmitted from poultry to humans. Up to date, WHO has reported approximately 60% mortality rate of confirmed human cases of recently emerged influenza A virus subtype H5N1 worldwide. In Thailand, there were three major outbreaks. The first one was in January 2004. The second and the third were in July 2004 and October 2005, respectively. At the end, there were 22 confirmed human cases with 14 fatalities (Amonsin et al., 2006). During the third outbreak, H5N1 viruses from plasma sample of infected human were isolated by embryonated egg inoculation as recommended by OIE. The isolate was designated as “A/Thailand/ NK165/2005” (Amonsin et al., 2006). Since live virus can be isolated from human blood sample, this is of great concern that the virus could genetically adapt or reassort with human transmissible viruses to efficiently transmit between human populations.

Although most patients with H5N1 infection died from acute respiratory distress (Chotpitayasunondh et al., 2005; de Jong et al., 2005; de Jong and Hien, 2006), cases with involvement of the central nervous system have also been reported (de jong et al., 2006; Maines et al., 2008). Infection with H5N1 isolated from Novasibirsk region caused changes in mouse brain structures, which include vasculopathies with thrombosis of the microcirculatory vessels, perivascular and pericellular edema with multifocal ischemic necrosis, hyperplasia of glial cells, caspase-dependent apoptosis of nerve cells and hypercytokinemia (Potapova et al., 2009). Similarly, H5N1 (A/Vietnam/1203/2004) has been shown to be neuroinfectious in a mouse model of H5N1 infection followed by robust glial activation and a significant loss of dopaminergic neurons of the midbrain (Jang et al., 2009). Recent study has identified the olfactory system could be a major route for brain invasion and, therefore, suggests that efficient growth of virus in the upper respiratory tract could facilitate viral brain invasion (Shinya et al., 2011). Several studies have provided evidence of acute encephalitis in humans infected with H5N1 viruses (de Jong et al., 2005). Postmortem analysis revealed that the virus was found in several parts of the brain with elevation of pro-inflammatory cytokines in the CSF (Gu et al., 2007; Thanawongnuwech et al., 2005). Moreover, it has been reported that the hemagglutinin protein of H5N1 (A/Vietnam/1203/2004) strongly induces pro inflammatory cytokine responses in human epithelial cells, as well as in human astrocytic and neuronal cell lines (Cheng et al., 2010; Ng et al., 2010). Based on these findings, H5N1 virus could induce profound injury to the central nervous system. However, molecular mechanism involved in this process remains elusive.

Procedures

All procedures involved with infection and handling of live influenza A virus subtype H5N1 (A/Thailand/NK165/2005) virus were performed exclusively in a biosafety level 3+ facility at the Faculty of Medicine, Chulalongkorn University, Thailand.

Virus propagation and hemagglutination titrations

Stocks of NK165 virus were kindly provided by Prof. Yong Poovorawan (Chulalongkorn University, Thailand). The virus was propagated in 12-day-old embryonated chicken eggs at 35°C for 3 days. Then, the eggs were stored overnight at 4°C before harvesting virus-containing allantoic fluids. Virus titer was determined by hemagglutination (HA) assay (Kistner et al., 2007). Briefly, the allantoic fluids were centrifuged at 10,000 x g for 10 min to obtain clear supernatant. Then, 25 µl of supernatant was mixed with 25 µl of phosphate buffered saline (PBS) in 96-well plate and serially diluted two-fold. Subsequently, 50 µl of 1% chicken red blood cell was added to each well and gently agitated. The plate was incubated for 30 min at room temperature. The last dilution showing complete agglutination of the red blood cells was counted and expressed as hemagglutination unit.

Cell culture and infection

Human neuroblastoma SH-SY5Y cells (ATCC, USA) were cultured in DMEM-F12 medium (HyClone, USA) supplemented with 10% fetal bovine serum (Gibco, USA), 100 U/ml of penicillin and 100 µg/ml of streptomycin at 37°C in 5% CO_2. Then, cells were infected with NK165 virus at a multiplicity of infection (MOI) of 1. After 1 h absorption, cells were washed with PBS and cultures were continued in 10% serum supplemented medium at 37°C under standard cell culture conditions. Mock-infected cells served as negative controls. Cell-free supernatants from infected and mock-infected cultures were collected at 0, 6, 12, 24, 48, and 72 h post-infection to determine the titer of progeny viruses by HA assay.

Cell viability assay

To access the viability of infected cells, the number of viable cells at different time points post-infection was determined using trypan blue dye exclusion assay. The assay is based on the ability of intact viable cells to exclude trypan blue dye. Briefly, NK165-infected SH-SY5Y and

mock-infected cells at 2 x 10^5 cells/well were cultured in 6-well plates. At determined time points post-infection, the cells were washed once with PBS and trypsinized to collect cell pellets by centrifuging at 300 x g for 5 min followed by re-suspending the pellet in PBS. Subsequently, 0.2 ml of the suspension were mixed with an equal volume of 0.4% trypan blue (Sigma, USA), incubated for 5 min and observed under a microscope to count total cell and stained cell numbers using a hemocytometer.

CPE assay

The cytopathic effects (CPEs) manifested by multinucleated giant cells, cell shrinkage and foci of cell destruction in SH-SY5Y cultures following NK165 virus infection were examined. The cultures were photographed using a phase-contrast microscope and the CPEs were scored as previously described (0, no CPE; +/-, enlargement of some cells in monolayer; 1+, 1-25% CPE; 2+, 25-50% CPE; 3+, 50-75% CPE; 4+, 75-100% CPE)(Moore et al., 1981).

Indirect immunofluorescence assay

Mock and NK165-infected SH-SY5Y cells on cover slips at different time points post-infection were washed with PBS and fixed with absolute methanol at room temperature for 20 min. Then, the cover slips were air dried for 30 min. Subsequently, cells were permeabilized with 0.3% Triton X-100 in PBS for 10 min at room temperature and washed with PBS. The cells were blocked with 5% normal goat serum made up in 0.03% Triton X-100/PBS for 1 h at room temperature before incubating with mouse monoclonal anti-H5N1 hemagglutinin (1:100, ProSci Inc., USA) at 4°C overnight. After washing with 0.03% Triton X-100/PBS, the cells were incubated with goat anti-mouse IgG (H+L) AlexaFlour 594-conjugated (1:5000; Invitrogen, USA) mixed with DAPI (1:500; Invitrogen, USA) for 1 h at room temperature. After extensively washing with 0.03% Triton X-100/PBS, the coverslips were mounted onto glass slides using anti-fade mounting media (Invitrogen,

USA). The stained cells were visualized using an Olympus inverted fluorescence microscope with DP2-BSW application software (Olympus Imaging America Inc., USA).

Preparation of membrane extract

SH-SY5Y cells were washed twice with Tris buffered saline (TBS). The cell suspension was collected and centrifuged at 1200 x g at 4°C for 4 min. The pellets were re-suspended in 1 ml of ice-cold buffer M containing 100 mM of NaCl, 20 mM of Tris-HCl (pH 8), 2 mM of $MgCl_2$, 1 mM of EDTA, 0.2% Triton X-100 and protease inhibitor cocktail (Pierce, USA). The pellets were lysed by vigorous vortex and centrifuged at 600 x g for 3 min at 4°C for separation of the nuclei and cell debris. The supernatant was collected, centrifuged at 6000 x g for 5 min at 4°C to collect the membranous organelles in the supernatant part and subsequently transferred to an ultracentrifuge tube and centrifuged at 20,800 x g for 10 min at 4°C to collect the membrane protein pellets. The pellets of membrane protein were resuspened in 50 ul of ice-cold buffer M (Sakoonwatanyoo et al., 2006). Protein concentration was measured by BCA assay kit (Pierce, USA).

1D-VOPBA and LC-MS/MS

Membrane proteins (100 ug) were separated on 10% SDS-PAGE and transferred onto PVDF membrane, followed by incubating with 5% skim milk in TBS at room temperature for 2 h. The NK165 virus was diluted with 1% skim milk in TBS and subsequently incubated for 2 h at room temperature or at 4°C. The amount of the virus used in the experiment was $1x10^7$ pfu/cm^2 of the membrane area. After washing three times with TBS, the PVDF membranes were incubated with a mouse monoclonal antibody directed against the H5N1 hemagglutinin protein (ProSci, USA) at a dilution of 1:200 in 5% skim milk in TBS at room temperature for 2 h. After incubation, the membranes were washed three times and then incubated with peroxidase-conjugated rabbit anti-mouse IgG at a dilution of 1:3000 in 5% skim milk in TBS, under constant agitation at room

temperature for 1h. After washing in TBS, the signal was generated by ECL-Plus Western Blotting Substrate (Pierce, USA) and directly exposed to CL-XPosure film (Pierce, USA). The other corresponding protein bands of SDS-PAGE were cut from the Coomassie blue-stained gel and the proteins interacting with NK165 virus were submitted to Genome Institute (BIOTEC, Thailand) for protein identification using liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS).

Western blot analysis

Cultures of mock and H5N1-infected SH-SY5Y cells were harvested at 24 h to collect cell pellets by centrifuging at 300 x g for 5 min. The pellets were resuspended in ice-cold RIPA buffer containing 150 mM of NaCl, 50 mM of Tris-HCl (pH 8), 0.5% DOC, 1 mM of EDTA, 0.2% Triton X-100, 1% SDS and protease inhibitor cocktail (Pierce, USA) and briefly sonicated on ice followed by centrifuging at 14000 x g for 10 min at 4°C to collect protein in supernatant. Protein concentration was measured by BCA assay (Pierce, USA). Whole cell proteins were separated by SDS-PAGE and transferred to PVDF membranes by electroblotting. The membrane were then blocked with 5% skim milk in TBS for 1 h at room temperature, then incubated with either a mouse monoclonal antibody directed against RACK1 protein (1:500; Santa Cruz, USA) or a goat polyclonal antibody directed against prohibitin protein (1:200; Santa Cruz, USA) made up in 5% skim milk in TBS overnight at 4°C. The blots were then washed three times with TBS and incubated with HRP-conjugated secondary Ab for 1 h at room temperature. Subsequently, after washing three times in Tris-buffered saline with 0.1% Tween 20, the signal was generated by ECL-Plus Western Blotting Substrate (Pierce, USA) and directly exposed to CL-XPosure film (Pierce, USA).

Data analysis

Data were presented as mean ± S.E.M. from experiments performed in triplicate in order to confirm the reproducibility of the results. Statistical comparisons; quantitative data of significance was done by a paired *t* test with a probability value of $p < 0.05$ was considered to be statistically significant.

Results

H5N1 induced CPEs in SH-SY5Y cells.

To examine neuroinfectious capacity of H5N1 (A/Thailand/ NK165/2005) in human nerve cells, human neuroblastoma SH-SY5Y cells were infected with NK165 at moi of 1 and compared to the mock-infected cells at 6, 12, 24 , 48 and 72 h post-infection. Cell undergoing cytopathic changes in each condition were photographed and scored, as shown in Fig. 1 and Table 1, respectively. CPEs in SH-SY5Y cells were characterized as follows: first, the cells became round and formed small aggregates or progressed from a typical neuronal morphology, extension of neurites to round dead cells with cell debris in the supernatant. CPEs were first observed in cultures of NK165-infected SH-SY5Y cells at 24 h post infection. The CPEs progressed rapidly and by 72 h post-infection entire cells in the cultures detached from the substratum. These findings demonstrated that H5N1/NK165 infection induces severe cytopathic effects in human neuroblastoma cells.

Permissiveness of human neuroblastoma SH-SY5Y cells to H5N1

To determine the course of H5N1 infection in SH-SY5Y cells, cells were infected with NK165 at MOI of 1. At 0, 6, 12, 24, 48 and 72 h post-infection, the presence of virus antigens were examined using immunofluorescent labeling of H5N1 specific hemagglutinin. Results demonstrated that the viruses were found in the cytoplasm of the infected cells as early as 12 h post-infection. By 24 h post-infection, all cells in the cultures were infected. The presence of virus antigen in the cell

debris was prominent at 72 h post-infection (Fig. 2). These findings coincided with time course of CPE reported in previous experiment. The presence of viruses in the cytoplasm strongly suggests that SH-SY5Y cells express the receptor for H5N1/NK165. Moreover, these results also suggest that human neuroblastoma cells support the replication of H5N1 virus.

To study the kinetics of viral production in human neuroblatoma cells, the presence of viral progeny in the media at different time points post-infection were determined using hemagglutination assay. Simultaneously, viability of the infected cell was assayed by trypan blue dye exclusion method. As showed in Fig. 3, results demonstrated that virus progeny released from the infected cells into the culture medium were first observed at 12 h post-infection. Virus titer increased exponentially overtime. After 24 h post-infection, viability of the infected cell began to decline. By 72 h, only cellular debris remained in the infected cultures. These results demonstrated that human neuroblatoma cells are permissive to H5N1/NK165 virus. Moreover, the results also suggest that pronounced death of the remaining cells in the infected cultures could be the consequence of recurrent infection by virus progeny.

H5N1-binding proteins on SH-SY5Y cells

To identify H5N1 virus binding proteins on human neuroblastoma SH-SY5Y cells, membrane-enriched samples were prepared and separated on SDS-PAGE and transferred to PVDF membrane. Then, the membranes were incubated with NK165 for 2 h at 4°C or at room temperature. The positions of virus-binding protein were visualized by successive incubations with a monoclonal antibody directed against H5N1 hemagglutinin protein and secondary HRP-conjugated rabbit anti-mouse IgG. Negative control without virus overlaid was also applied in parallel. Results were shown in Fig. 4. There were several virus-binding protein bands observed, if the membranes were incubated with the virus at room temperature compared with that of 4°C. However, three specific bands at the approximately 36-38 kDa, 34-35 kDa and 28-30 kDa appeared at both incubation temperatures were

excised and processed for LC-MS/MS analysis. Results were showed in Table 2, which indicates number of fraction and size range in molecular mass (kDa), names of H5N1-binding protein candidates, its predicted mass, accession number of protein sequences searched on the NCBInr database and % coverage of peptide match. RACK1 and prohibitin were selected as H5N1-binding proteins based on their molecular masses, % coverage of the peptide match and subcellular localization of these proteins. Expression of RACK1 and prohibitin in mock and NK165-infected SH-SY5Y cells were confirmed by Western blot. Results were shown in Figure 5. Both RACK1 and prohibitin were normally expressed by SH-SY5Y cells. Moreover, at 24 h post-infection there was a significant decrease in expression of RACK1 and prohibitin proteins compared with mock-infected cells. These findings suggest that both RACK1 and prohibitin might be involved in the initial step of H5N1 binding to the nerve cell membrane and/or internalization.

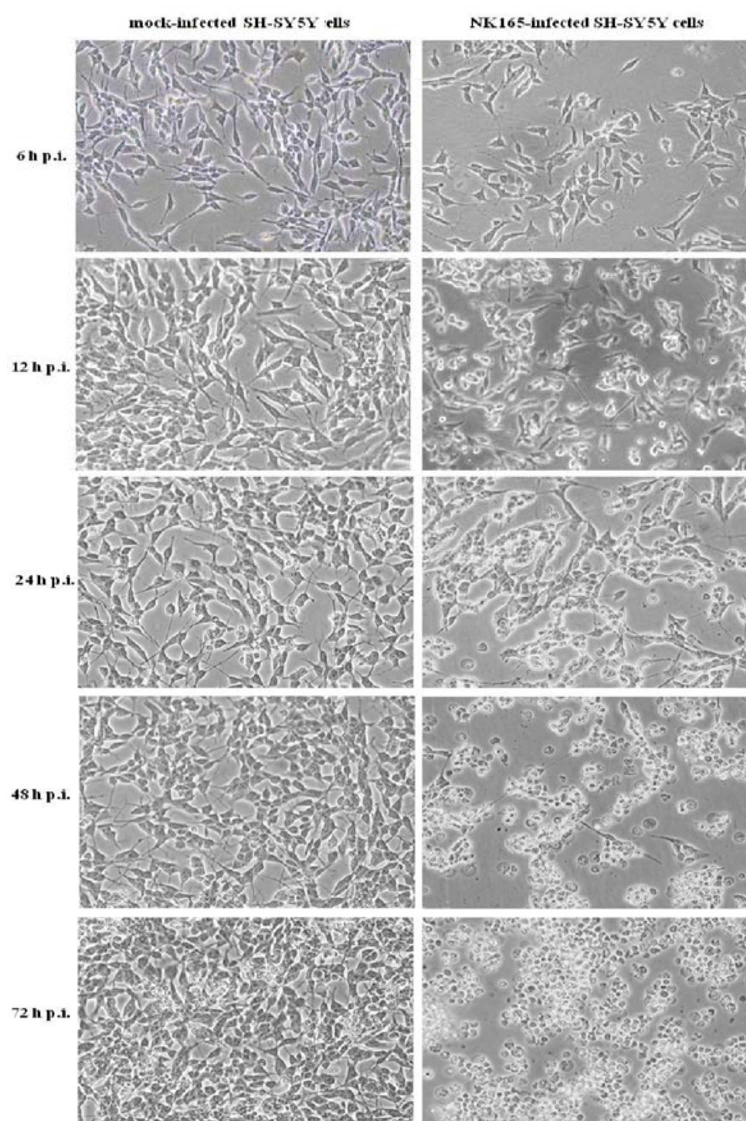

Fig. 1. Cytopathic effects (CPE) of SH-SY5Y cells infected with influenza A H5N1 virus (Thailand/NK165/05) at MOI of 1. At 6, 12, 24, 48 and 72 h post-infection (p.i.). The morphology of the infected cells were compared with that of the mock-infected cells. Representative photomicrographs taken at 200x magnification showed progressive morphological changes with increasing small cell aggregation and cellular debris.

Table 1. CPE score of H5N1-infected SH-SY5Y cells at indicated time points

CPEs Scoring	0	+/-	1+	2+	3+	4+
Mock infected cells in all conditions	√					
6 h p.i.	√					
12 h p.i.		√				
24 h p.i.			√			
48 h p.i.					√	
72 h p.i.						√

(0 = no CPE; +/- = enlargement of some cells in the monolayer; 1+ = 1-25% CPE; 2+ = 25-50% CPE; 3+ = 50-75% CPE; 4+ = 75-100% CPE, as described previously (Moore et al., 1981)

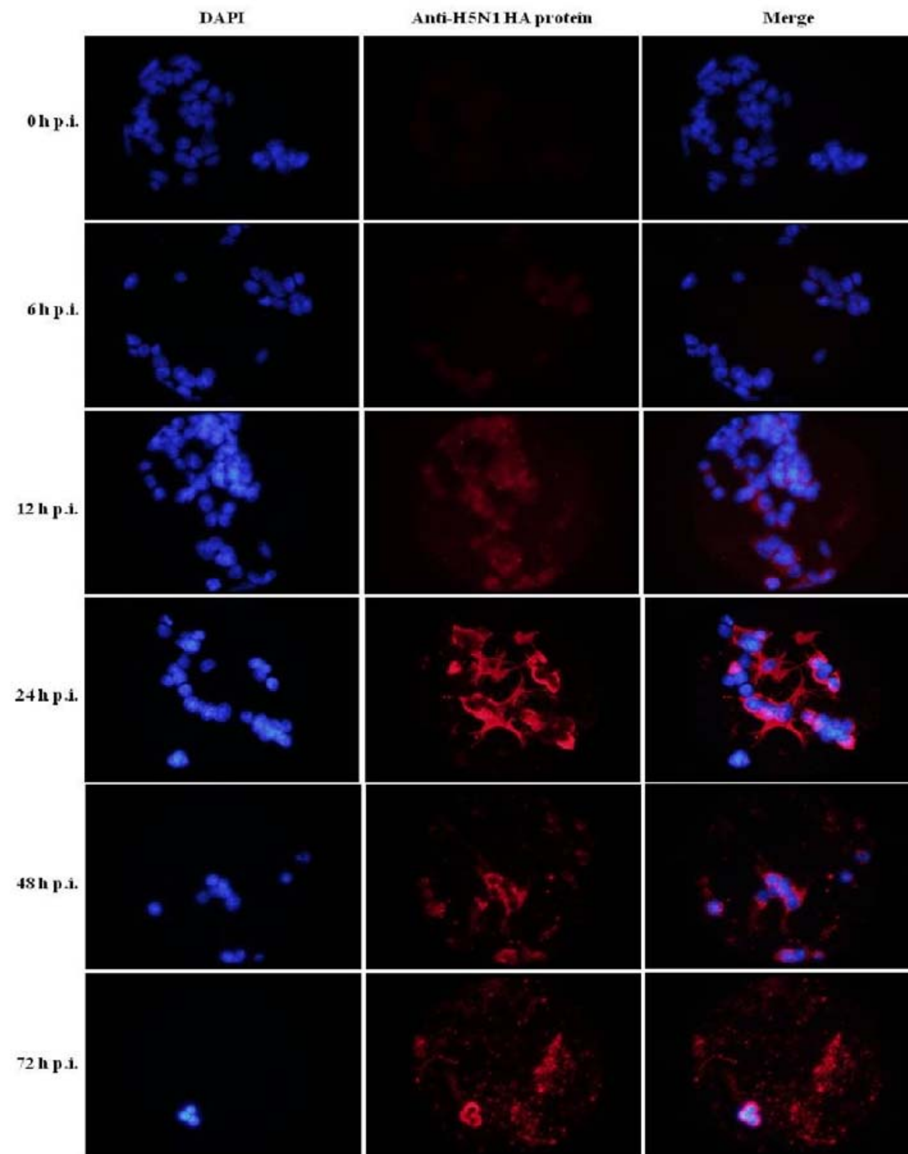

Fig.2. Immunofluorescence staining of SH-SY5Y cells infected with H5N1 virus. Cells were infected with the virus at MOI of 1 at indicated time points and were examined for the presence of H5N1 specific hemagglutinin (HA) using a mouse monoclonal anti-H5N1 HA (red). The nuclei were counterstained with DAPI (blue). Representative photomicrographs taken at 400x magnification were showed.

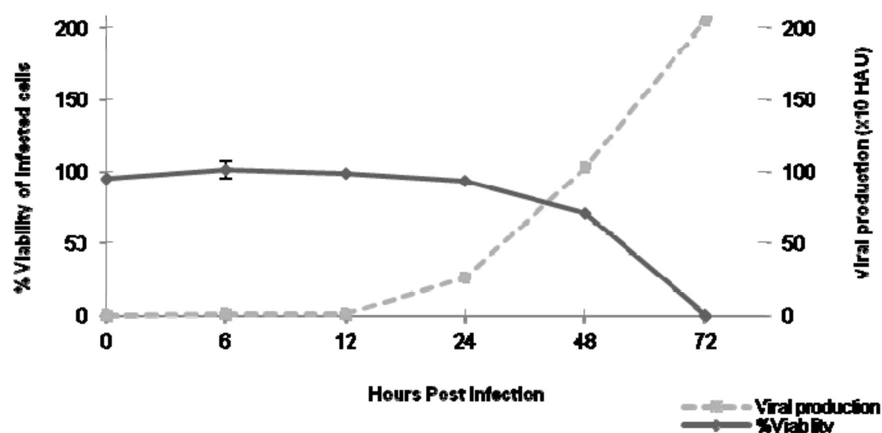

Fig. 3. Virus production and susceptibility of SH-SY5Y cells to H5N1 infection. Confluent monolayers of SH-SY5Y cells were infected with H5N1 virus at MOI of 1. At indicated time points, cell-free supernatants were collected for hemagglutination assay. The viability of mock and infected cells was determined by trypan blue dye exclusion method. All experiments were performed in triplicate. The dashed and solid lines indicate the amount of virus produced and infected cell viability compared with mock infection, respectively.

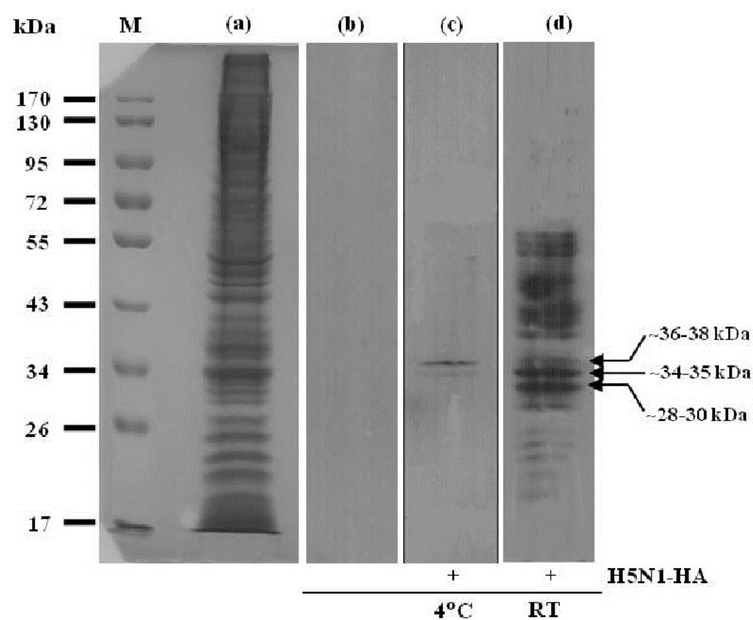

Fig. 4. Virus overlay protein binding assay (VOPBA) reveals H5N1 virus binding proteins on SH-SY5Y cells. Membrane proteins enriched were prepared from confluent monolayer of SH-SY5Y cells, separated by SDS-PAGE and stained by coomassie blue dye (a). After transferred to PVDF membranes, proteins were incubated with H5N1 virus at 4°C (c) or room temperature (d). Positions of H5N1 virus binding proteins were specifically detected using a mouse monoclonal anti-H5N1 HA. Simultaneously, negative controls without virus incubation step at 4°C or room temperature were performed in parallel (b). Molecular weight protein markers are indicated (M).

Table 2. The candidate H5N1 virus-binding proteins on SH-SY5Y cells identified by LC-MS/MS.

Fraction (size range in kDa)	Accession no.	Protein name	Location[a]	Function/structure in which protein is involved[b]	Mol mass (Da)	% Coverage
1 (~36-38)	NP_002128.1	Heterogeneous nuclear ribonucleoprotein A2/B1 isoform A2	N, C	pre-mRNA processing, mRNA metabolism and transport	36005.7	60.70
	NP_060979.2	Leucine rich repeat containing 59	ER, Mi, M	Protein-protein interactions and have different functions and cellular locations	34930.2	37.13
2 (~34-35)	sp|O42249.1	Guanine nucleotide-binding protein subunit beta-2-like 1 (Receptor of activated protein kinase C) (RACK1)*	C, M	Intracellular receptor to anchor the activated PKC to the cytoskeleton, adaptor/regulatory modules in signal transduction, pre-mRNA processing and cytoskeleton assembly	35076.5	84.86
	sp|P40926	MDHM_HUMAN Malate dehydrogenase, mitochondrial precursor	Mi	L-malate dehydrogenase activity	35531.1	56.21
	sp|Q16836	HCDH_HUMAN Hydroxyacyl-coenzyme A dehydrogenase, mitochondrial precursor (Short chain 3-hydroxyacyl-CoA)	Mi	Plays an essential role in the mitochondrial beta-oxidation of short chain fatty acids	34277.2	10.51
	NP_009204.1	Prohibitin 2	Mi, C, N	A mediator of transcriptional repression by nuclear hormone receptors via recruitment of histone deacetylases	33296.1	10.51
3 (~28-30)	NP_002625.1	Prohibitin*	Mi, N, M	Inhibits DNA synthesis, It has a role in regulating proliferation, as yet it is unclear if the protein or the mRNA exhibits this effect	29803.9	70.96
	NP_004729.1	VAMP-associated protein B/C	G, ER, M	Vesicle trafficking	27228.2	30.40
	sp|Q9Y5M8	SRPRB_HUMAN Signal recognition particle receptor subunit beta (SR-beta) (Protein APMCF1)	C, ER, M	Mediate the membrane association of SR-alpha	29702.0	29.90
	ssp|P05141	ADT2_HUMAN ADP/ATP translocase 2 (Adenine nucleotide translocator 2) (ANT 2) (ADP,ATP carrier protein 2)	Mi, M	Catalyzes the exchange of ADP and ATP across the mitochondrial inner membrane	32895.0	22.10

[a] The locations of the protein are indicated as follows: C, cytoplasmic (can include intracellular vesicles); M, plasma membrane or integral to plasma membrane; N, nuclear; ER, endoplasmic reticulum; G, Golgi; Mi, mitochondrion

[b]Data from NCBI Protein Databases.

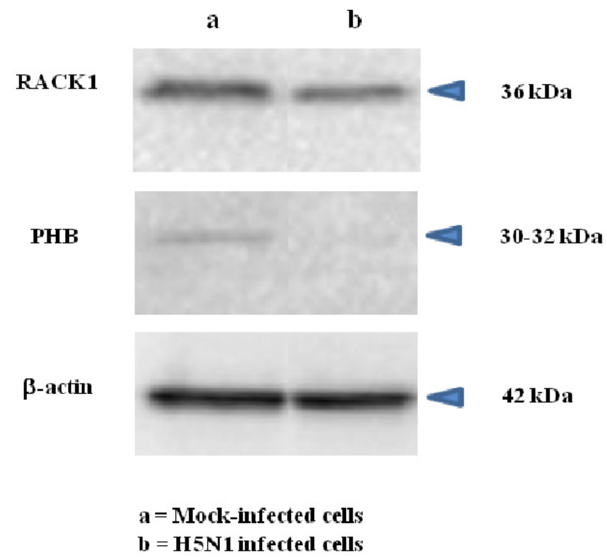

Fig. 5. Western blot analysis of RACK1 and prohibitin (PHB) expression in mock (a) and H5N1-infected (b) SH-SY5Y cells at 24 h post-infection.

Discussion

To explore neuroinvasiveness of H5N1 virus in human, this study employed the isolate designated as A/Thailand/NK165/2005 that caused the third outbreak in Thailand to infect human neuroblastoma SH-SY5Y cell line. This particular cell line is widely used as an in vitro model of mesencephalic neurons for Parkinson's disease research (Xie et al., 2010). Our results indicate that NK165 is highly pathogenic to human nerve cells. Using immunofluorecence staining technique, H5N1-specific hemagglutinin was detected in neuronal cells as early as early as 12 h post-infection coinciding with the time course observed for CPE. We also showed that neuronal infection occurred rapidly within 24 h. The infection was progressive leading to destruction of neuronal cells maintained in cultures within 72 h. Since not all cells at the beginning of the infection were infected, we therefore propose that virus progeny released from the infected cells would spread and reinfect the remaining cells. Our findings also suggest two folds: the presence of viral receptor on neuronal cell membrane and replication machinery that serve viral replication in neuronal cells.

Studies in the brains of mice infected by H5N1 have demonstrated that neurotropic H5N1 viruses are predominantly localized in the basal ganglia after reaching the brainstem solitary nucleus, which receives primary afferent signals from the visceral organs (Jang et al., 2009). These authors also reported a significant loss of dopaminergic neurons in the substantia nigra of the infected mouse brains consistent with their neurological symptoms including ataxia, tremor, and bradykinesia. Thus, these findings suggest that mesencephalic neurons are the prime target of H5N1. In the present study, we provided direct evidence that human mesecephalic neurons can be infected by H5N1 virus. We have demonstrated that H5N1/NK165 virus is capable of replicating in human mesencephalic SH-SY5Y cells, which is in line with the recent report that NK165 and is in the same clade with neurotropic H5N1 A/Vietnam/1203/04 virus (Babakir-Mina et al., 2009). Thus, individuals infected by NK165, if survived long enough, could therefore succumb from subsequent neurological disorder

including post-encephalitic parkinsonism similar to the notorious Spanish flu (influenza A virus subtype H1N1) that caused the great 1918 pandemic and gave rise to global outbreak of encephalitis and its neurological sequelae.

Although there are several reports stating that H5N1 virus can invade the brain and deliberate encephalopathy or acute encephalitis. (de Jong et al., 2005; Jang et al., 2009), till date candidate neuronal proteins that may be involved in brain infection has not yet been determined. Therefore, knowledge at the molecular level as how to the neurotropic strain of H5N1 virus interacts with nerve cells is critical. Using a combination of 1D-VOPBA and LC-MS/MS analysis, we have identified RACK1 and prohibitin as neuronal receptors for H5N1 virus.

It has been reported that M1 protein of the influenza A virus interacts with the cellular receptor of activated C kinase (RACK) 1 and can be phosphorylated by protein kinase C (PKC). The M1-RACK1 interaction is of general importance during avian, swine and human influenza A virus infections (Reinhardt and Wolff, 2000). Prohibitin has been reported to serve as a receptor that mediats DENV-2 entry into insect cells (Kuadkitkan et al., 2010). Prohibitin is ubiquitously expressed in eukaryotic cells (Morrow and Parton, 2005). It is present in many cellular compartments, mainly mitochondria (Ikonen et al., 1995; Merkwirth and Langer, 2009; Nijtmans et al., 2000) but also in the cytoplasm, nucleus (Thompson et al., 2001; Wang et al., 2002; Wang, Zhang, and Faller, 2002) and cell membrane (Kolonin et al., 2004; Sharma and Qadri, 2004). Our results showed that the absence of prohibitin in H5N1-infected SH-SY5Y cells suggesting that prohibitin could be down-regulated during virus internalization. This process may be implicated in modulating the intensity of cell surface protein receptors on infected cells. A similar strategy is also utilized by HIV-1 in the CD4 down-modulation during infection (Chen et al., 1996).

Conclusion

We have demonstrated that H5N1 A/Thailand/NK165/2005 /NK165 is neuroinvasive to human. We also identified RACK1 and prohibitin as potential viral receptors on neuronal cell menbrane. However, further studies are needed to explore whether both RACK1 and prohibitin are actual receptors for H5N1. This will ultimately lead to improved therapeutics and provide an insight into neuronal mechanism of H5N1 infection.

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