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N-acetyl-L-cysteine (NAC) inhibits virus replication and expression of pro-inflammatory molecules in A549 cells infected with highly pathogenic H5N1 influenza A virus

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ABSTRACT

The antioxidant N-acetyl-L-cysteine (NAC) had been shown to inhibit replication of seasonal human influenza A viruses. Here, the effects of NAC on virus replication, virus-induced pro-inflammatory responses and virus-induced apoptosis were investigated in H5N1-infected lung epithelial (A549) cells. NAC at concentrations ranging from 5 to 15 mM reduced H5N1-induced cytopathogenic effects (CPEs), virus-induced apoptosis and infectious viral yields 24 h post-infection. NAC also decreased the production of pro-inflammatory molecules (CXCL8, CXCL10, CCL5 and interleukin-6 (IL-6)) in H5N1-infected A549 cells and reduced monocyte migration towards supernatants of H5N1-infected A549 cells. The antiviral and anti-inflammatory mechanisms of NAC included inhibition of activation of oxidant sensitive pathways including transcription factor NF-κB and mitogen activated protein kinase p38. Pharmacological inhibitors of NF-κB (BAY 11-7085) or p38 (SB203580) exerted similar effects like those determined for NAC in H5N1-infected cells. The combination of BAY 11-7085 and SB203580 resulted in increased inhibitory effects on virus replication and production of pro-inflammatory molecules relative to either single treatment. NAC inhibits H5N1 replication and H5N1-induced production of pro-inflammatory molecules. Therefore, antioxidants like NAC represent a potential additional treatment option that could be considered in the case of an influenza A virus pandemic.

1. Introduction

Highly pathogenic H5N1 influenza A viruses are considered to be potential progenitors of a novel influenza pandemic [1–6]. Human infections with highly pathogenic influenza A H5N1 viruses are associated with severe pneumonia, lymphopenia, high viral loads in the respiratory tract, and hyper-induction of cytokines and chemokines (cytokine storm) [7]. Pathological investigations revealed that induction of apoptosis may be a major mechanism in the destruction of alveolar epithelial cells in humans infected with H5N1 [8]. Apoptosis may be a direct consequence of virus replication as well as result from excessive inflammatory responses to virus infection [6].

Production of reactive oxygen species (ROS) has been shown to contribute to pulmonary damage caused by influenza virus infection [9–11]. Recently, ROS were suggested to contribute to acute lung injury in people with severe influenza A virus infection by triggering the signalling of oxidised phospholipids through toll-like receptor 4 (TLR4)-TIR-domain-containing adaptor-inducing interferon-β (TRIF)-TNF receptor associated factor 6 (TRAF6) cascade [12,13]. Different sources of ROS have been suggested in influenza A virus-infected lungs. Leukocytes may be activated and primed by influenza A virus infection and produce ROS [14]. Moreover, increased xanthine oxidase levels were found in influenza A virus-infected lungs [14]. Epithelial cells of the lungs may also be a source of ROS since influenza A virus infection-induced oxidant stress response in cultured airway epithelial [15,16].

Endogenous oxidants may be involved in signal transduction pathways that stimulate production of cytokines/chemokines through activation of transcription factors and induction of pro-inflammatory gene expression in influenza A-infected cells [16]. Influenza A viruses including H5N1 strains were shown to induce expression of cytokines and chemokines, including CXCL8 (also known as interleukin-8), interleukin-6 (IL-6), CXCL10 (also known as interferon-inducible cytokine IP-10), and CCL5 (also known as RANTES) in macrophages and airway epithelial cells through

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oxidant sensitive pathways such as mitogen activated kinase p38 and the transcription factor nuclear factor-κB (NF-κB) [2,17–19].

Antioxidant molecules including reduced glutathione and its precursor N-acetyl-L-cysteine (NAC) are potentially useful against infection with influenza A viruses [20–23]. Notably, NAC was already shown to synergise with oseltamivir in the treatment of lethal seasonal influenza A virus infection in a mouse model [23]. Here, we investigated the effects of NAC as prototype antioxidant on virus replication, virus-induced apoptosis, and expression of pro-inflammatory molecules in A549 cells infected with human H5N1 influenza A virus. Moreover, the influence of NAC on NF-κB and p38, both constituents of cellular signalling pathways known to be of relevance for influenza A virus replication, was studied in H5N1-infected cells.

2. Materials and methods

2.1. Virus stock

The H5N1 influenza A strain A/Thailand/1(Kan-1)/04 was obtained from Prof. Pilaipan Puthavathana (Mahidol University, Bangkok). The H5N1 influenza A strain A/Vietnam/1203/04 was received from the WHO Influenza Centre at the National Institute for Medical Research London (Great Britain). Virus stocks were prepared by infecting Vero cells (African green monkey kidney; ATCC: CCL81, Manassas, VA, USA) and aliquots were stored at −80 °C. Virus titres were determined as 50% tissue culture infectious dose ($TCID_{50}$/ml) in confluent cells in 96-well microtitre plates (Greiner Bio-One, Frickenhausen, Germany).

2.2. Cells

A549 cells (human lung carcinoma; ATCC: CCL185, Manassas, VA, USA) and Vero cells were grown at 37 °C in minimal essential medium (MEM, Biochrom AG, Berlin, Germany) supplemented with 10% foetal bovine serum (FBS, Sigma–Aldrich Chemie GmbH, Munich, Germany), 100 IU/ml penicillin (Grünethal GmbH, Aachen, Germany) and 100 μg/ml streptomycin (Sigma–Aldrich Chemie GmbH, Munich, Germany).

2.3. Drugs

N-acetyl-L-cysteine (NAC) was obtained from Alexis (distributed by Axxora, Germany), dissolved in unsupplemented MEM and adjusted to pH 7.4 with NaOH. The caspase-3 inhibitor Ac-DEVD-CHO, the NF-κB inhibitor BAY 11-7085 and the p38 MAP kinase inhibitor SB203580 were obtained from Merck Biosciences (Darmstadt, Germany).

2.4. Cell viability assay

Confluent A549 cells were treated with NAC for 48 h. The cellular viability was assessed with CellTiter-Glo® Luminescent Cell Viability Assay (Promega GmbH, Mannheim, Germany) according to the manufacturers' protocol. Cell viability was expressed as percentage of non-treated control.

2.5. Cytopathogenic effect (CPE) reduction assay

Confluent A549 cell layers were infected with influenza A (H5N1) at a MOI of 0.01 in MEM supplemented with 2% FBS, 100 IU/ml penicillin and 100 μg/ml streptomycin. Cells were continuously treated with NAC starting with a 24 h pre-incubation period prior to infection. Twenty four hours post-infection (p.i.) the virus-induced CPE was recorded using an inverted light microscope (Olympus, Planegg, Germany).

2.6. Virus yield reduction assay

Confluent A549 cell layers were infected with influenza A (H5N1) at a MOI of 0.01 in MEM supplemented with 2% FBS, 100 IU/ml penicillin and 100 μg/ml streptomycin. Cells were continuously treated with NAC starting with a 24 h pre-incubation period prior to infection. At the given time points, aliquots of the supernatants were taken and serial 10-fold dilution steps were performed. Infectivity was determined by endpoint dilution titration onto Vero cells in 96-well microtitre plates. Plates were incubated for 3–4 days and infectivity was analysed by virus-induced cytopathogenic effect. Virus titres were calculated by the method of Reed and Muench [24].

2.7. Indirect immunofluorescence microscopy

Confluent A549 cell layers infected with influenza A (H5N1) at a MOI of 0.1 were treated with NAC (15 mM) or caspase-3 inhibitor I (20 μM, non-toxic concentration, data not shown). Cells were continuously treated with NAC or Ac-DEVD-CHO starting with a 24 h or 1 h pre-incubation period, respectively, prior to infection. Eight hours p.i., cells were fixed for 15 min with ice-cold acetone/methanol (40:60, Mallinckrodt Baker B.V., Deventer, The Netherlands) and stained with a mouse monoclonal antibody (1 h incubation, 1:1000 in PBS) directed against the influenza A virus nucleoprotein (NP) (Millipore, Molsheim, France). As secondary antibody an Alexa Fluor 488 goat anti-mouse IgG (H&L) (Invitrogen, Eugene, Oregon, USA) was used (1 h incubation, 1:1000 in PBS). Nuclei were stained using 4′,6-diamidino-2-phenylindole (DAPI) (Sigma–Aldrich Chemie GmbH, Munich, Germany). Fluorescence was visualised using Olympus IX 1 fluorescence microscope (Olympus, Planegg, Germany).

2.8. Isolation of human monocytes

Human monocytes were isolated from buffy coats of healthy donors, obtained from Institute of Transfusion Medicine and Immune Haematology, German Red Cross Blood Donor Center, Johann Wolfgang Goethe-University, Frankfurt am Main. After centrifugation on Ficoll (Biocoll)-Hypaque density gradient (Biochrom AG, Berlin, Germany) mononuclear cells were collected from the interface and washed with PBS (Sigma–Aldrich Chemie GmbH, Munich, Germany). Then, monocytes were isolated using magnetically labeled CD14 MicroBeads (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) following the manufacturer's instructions. Monocytes were cultivated in IMDM supplemented with 10% pooled human serum, 100 IU/ml of penicillin, and 100 μg/ml streptomycin.

2.9. Migration assay

Cell culture supernatants were investigated for chemotactic activity by measurement of the activity to induce monocyte migration through membrane inserts in 24-well plates (pore size 8 μm; BD Biosciences, Heidelberg, Germany). Monocytes (1×10^6 in 100 μl of IMDM with 10% pooled human serum) were added into the cell culture inserts (upper chamber) and cell culture supernatants (300 μl) were added to the lower chamber of the well. After a 48 h incubation period, cells were fixed with 4% paraformaldehyde and permeabilised with PBS containing 0.3% Triton X-100. Then, nuclei were stained with DAPI. The upper side of the membrane was wiped with a wet swab to remove the cells, while the lower side of the membrane was rinsed with PBS. The number of cells at the lower side of each membrane was quantified by counting of cells from three randomly chosen sections (3.7 mm^2) using an Olympus IX 1 fluorescence microscope.

2.10. Cytokine/chemokine secretion

Cell culture supernatants were collected and frozen at −80 °C. Cytokines/chemokines were quantified by specific ELISA Duo Sets (R&D Systems GmbH, Wiesbaden, Germany) following the manufacturer's instructions.

2.11. NF-κB activity

NF-κB activity was investigated by quantification of the NF-κB subunits Rel A (p65) and NF-κB1 (p50) from nuclear extracts using the NF-κB Transcription Factor assay kit (Active Motif, Rixensart, Belgium).

2.12. Western blot analysis

Cells were lysed in Triton X-sample buffer and separated by SDS-PAGE. Proteins were detected using specific antibodies against β-actin (Sigma–Aldrich, Taufkirchen, Germany), p38 (anti-p38 MAP kinase Ab, New England Biolabs GmbH, Frankfurt am Main, Germany) or phosphorylated p38 (anti-phospho-specific p38 MAP kinase Ab, New England Biolabs GmbH, Frankfurt am Main, Germany) and were visualised by enhanced chemiluminescence using a commercially available kit (Amersham, Freiburg, Germany).

2.13. Caspase activity

Cells were tested for caspase activity (expressed as relative luminescence units (RLUs)) using the Caspase-Glo®-3/7, -8, and -9 Assay kit (Promega GmbH, Mannheim, Germany) following the manufacturer's instructions. Luminescence was measured using TECAN Infinite 200 (Tecan Deutschland GmbH, Crailsheim, Germany).

2.14. Statistical analysis

All data are given as the mean ± SD. Statistical analysis of results was performed by Student's *t*-test. *P* values of <0.05 were considered statistically significant.

3. Results

3.1. Influence of NAC on H5N1 virus replication in A549 cells

To investigate effects of NAC on influenza A H5N1 virus replication in A549 cells, confluent cell layers infected with A/Thailand/1(Kan-1)/04 (Kan-1) or A/Vietnam/1203/04 (VN1203) at a MOI of 0.01 were treated with NAC at concentrations ranging from 5 to 15 mM. Cells were continuously treated with NAC starting with a 24 h pre-incubation period prior to infection (if not stated otherwise cells were treated like this throughout all experiments described in this report). Supernatants were collected 12, 24, or 48 h post-infection (p.i.) and virus titres were determined as $TCID_{50}$/ml. NAC reduced the titres of influenza A/Thailand/1(Kan-1)/04 in a concentration-dependent manner (Fig. 1A). Twelve hours post-infection (p.i.), no significant decrease of virus titres was observed. Treatment with 10 mM NAC significantly decreased the Kan-1 virus titre about 5.6-fold (24 h p.i.) and about 2.1-fold (48 h p.i.) compared to mock-treated virus control. Treatment with 15 mM NAC reduced the titres about 34.5-fold (24 h p.i.) and 5.8-fold (48 h p.i.). Similar results were obtained with A/Vietnam/1203/04. Significant reduction of virus replication was observed after treatment with 15 mM NAC 12 h p.i. (2.2-fold reduction), 24 h p.i. (48.9-fold reduction) and 48 h p.i. (25.2-fold reduction) and after treatment with 10 mM and 5 mM NAC 48 h p.i. (7.1-fold and 2.2-fold reduction). In addition, treatment of H5N1 (Kan-1)-infected cells with NAC (15 mM) almost completely suppressed formation of cytopathogenic effects (CPEs) (Fig. 1B). None of the tested NAC concentrations affected A549 cell viability (data not shown). Further experiments were performed with the influenza A strain A/Thailand/1(Kan-1)/04.

3.2. Influence of NAC on H5N1-induced caspase activation

ROS formation may result in caspase-dependent apoptosis and virus-induced apoptosis may be a major mechanism in the destruction of epithelial cells infected with H5N1 both *in vitro* and *in vivo* [8,25]. To investigate the influence of NAC on H5N1-induced apoptosis in A549 cells, cells were treated with or without NAC and infected with H5N1 at a MOI of 0.01. Twenty four hours

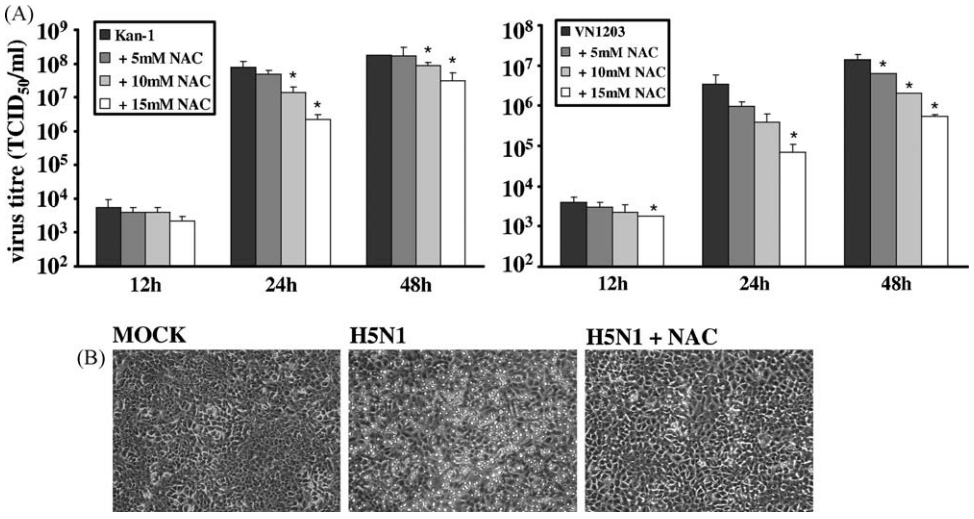

Fig. 1. Influence of N-acetyl-L-cysteine (NAC) on H5N1 virus replication and cytopathogenic effect (CPE) formation in A549 cells. (a) A549 cells were infected with A/Thailand/1(Kan-1)/04 (Kan-1) or A/Vietnam/1203/04 (VN1203) at a MOI of 0.01. NAC treatment (0 mM NAC: dark grey bars, 5 mM NAC: middle grey bars, 10 mM NAC: light grey bars, 15 mM NAC: white bars) was performed continuously starting 24 h prior to infection. H5N1 titres were determined 12, 24 and 48 h post-infection. Data represent the mean ± SD of three independent experiments. (b) H5N1-induced formation of CPE in A549 cells at 24 h post-infection. Representative photographs show non-infected cells (mock), cells infected with H5N1 strain A/Thailand/1(Kan-1)/04 at a MOI of 0.01, or H5N1-infected cells continuously treated with NAC 15 mM starting 24 h prior to infection. *$P < 0.05$ relative to untreated virus control.

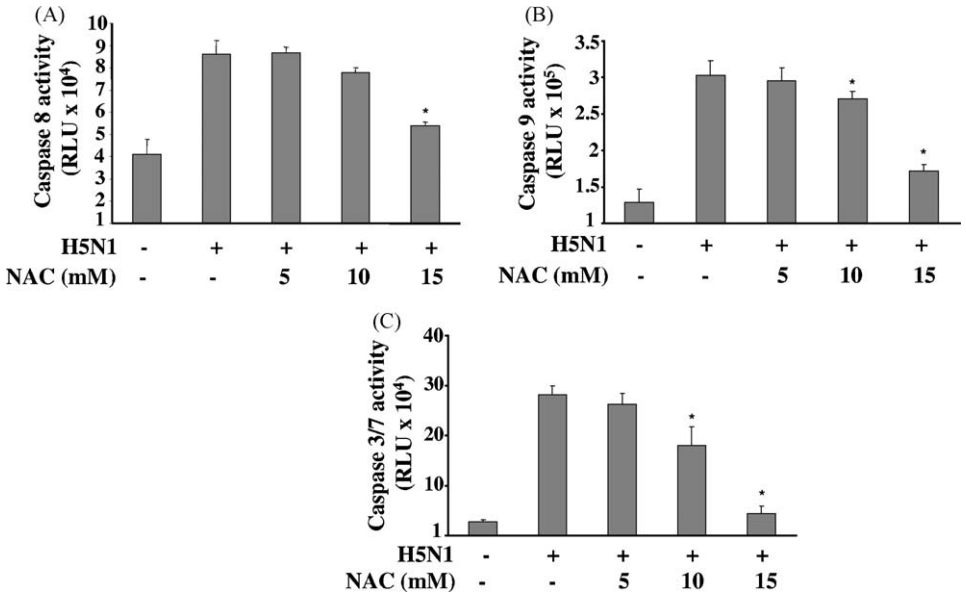

Fig. 2. Influence of N-acetyl-L-cysteine (NAC) treatment on caspase activation in H5N1-infected A549 cells. A549 cells were infected with A/Thailand/1(Kan-1)/04 (H5N1) at a MOI of 0.01. NAC treatment was performed continuously starting 24 h prior to infection. Twenty four hours post-infection cells were analysed for (a) caspase-8 (b) caspase-9 and (c) caspase-3/7 activity (expressed as relative luminescence units (RLUs)) using Caspase-Glo® Assay kit. Data represent the mean ± SD of 3 separate experiments. $^{*}P < 0.05$ relative to untreated virus control.

p.i. cells were analysed for caspase-8, -9 and -3/7 activity. H5N1 infection increased the caspase-8 (2.1-fold), caspase-9 (2.4-fold) and caspase-3/7 (7.7-fold) activity relative to the mock-infected cells (Fig. 2A–C). In H5N1-infected cells, NAC reduced activities of all investigated caspases in a dose-dependent manner. Compared to virus control, NAC 15 mM treatment decreased caspase-8 activity by 1.6-fold, caspase-9 activity by 1.8-fold, and caspase-3/7 activity by 5.4-fold.

Moreover, inhibition of caspase-3 resulted in retention of influenza virus ribonucleoprotein (RNP) complexes in the nucleus of infected cells and in turn to inhibition of influenza A virus replication [26]. To investigate the influence of NAC on retention of RNP complexes, A549 cells were treated with or without 15 mM NAC or caspase-3 inhibitor and infected with H5N1 (MOI 0.1). Eight hours p.i. cells were analysed for RNP export. Similar to the caspase-3 inhibitor that served as positive control, NAC inhibited nuclear export of the viral RNP as indicated by immunofluorescence staining (Fig. 3).

3.3. Influence of NAC on cytokine and chemokine production in H5N1-infected cells

Virus-induced cytokine/chemokine storm seems to contribute to severe pathogenesis of H5N1 infection in humans [6]. In

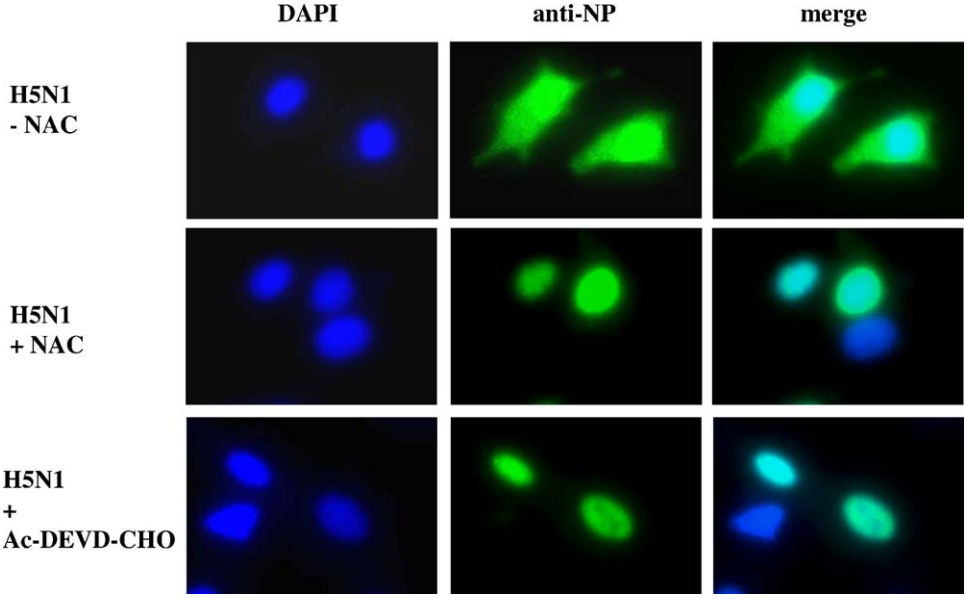

Fig. 3. Influence of N-acetyl-L-cysteine (NAC) treatment on nuclear export of viral NP in H5N1-infected A549 cells. A549 cells were infected with A/Thailand/1(Kan-1)/04 (H5N1) at a MOI of 0.1. NAC 15 mM treatment was performed continuously starting 24 h prior to infection. Eight hours post-infection NP localisation was visualised using specific antibodies by immunfluorescence. NP staining is shown in green. Nuclei are stained by DAPI (shown in blue). Since caspase-3 inhibition is known to block nuclear export of NP in influenza A virus-infected cells, the Caspase-3 inhibitor Ac-DEVD-CHO (20 μM, continuously treatment starting 1 h prior infection) was used as positive control. Photographs are taken from one representative experiment. In total, three independent experiments were performed with similar results.

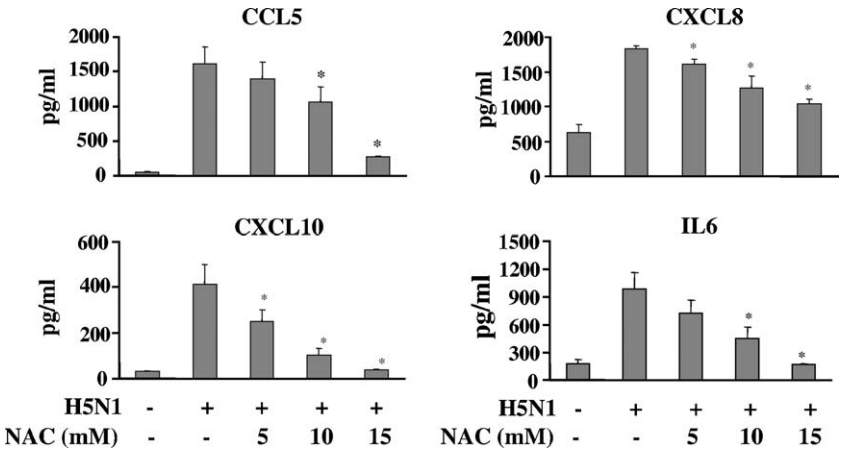

Fig. 4. Influence of N-acetyl-L-cysteine (NAC) treatment on production of cytokines/chemokines in H5N1-infected A549. A549 cells were infected with A/Thailand/1(Kan-1)/04 (H5N1) at a MOI of 0.01. NAC treatment was performed continuously starting 24 h prior to infection. Twenty four hours post-infection supernatants were analysed for CCL5, CXCL8, CXCL10, or IL-6 using ELISA. Data represent the mean ± SD of three separate experiments. $^*P < 0.05$ relative to untreated virus control.

addition to investigation of virus inhibitory effects, the influence of NAC on the production of cytokines/chemokines which had been correlated to progression of H5N1 disease was studied. Supernatants from mock- or H5N1-infected cultures were compared for expression of CCL5, IL-8, CXCL10 and IL-6 by ELISA (Fig. 4). Mock-infected cells produced low levels of all cytokines/chemokines tested. Basic levels were 53.6 ± 8.6 pg/ml for CCL5, 627.8 ± 119.7 pg/ml for CXCL8, 26.4 ± 10.4 pg/ml for CXCL10 and 156.8 ± 43.5 pg/ml for IL-6. H5N1 infection increased production of CCL5 by 30.0-fold (1607 ± 240.4 pg/ml), of CXCL8 by 2.9-fold (1826 ± 47.2 pg/ml), of CXCL10 by 15.6-fold (412.1 ± 85.7 pg/ml), and of IL-6 by 6.3-fold (985.5 ± 170 pg/ml). NAC did not significantly influence basal cytokine/chemokine levels in the investigated concentrations up to 15 mM (data not shown). The H5N1-induced cytokine secretion was reduced by NAC in a dose-dependent manner. Fifteen mM NAC could reduce the CXCL10 and IL-6 secretion to levels of untreated cells (mock) whereas the production of CXCL8 or CCL5 was significantly decreased in comparison to untreated H5N1-infected cells (1.7-fold or 6.0-fold reduction, respectively) but clearly remained higher than the mock levels (Fig. 4).

3.4. Influence of NAC on chemoattraction of monocytes by supernatants from H5N1-infected A549 cells

H5N1 infection is characterised by massive infiltration of monocytes/macrophages into the lungs in humans [6]. The potential to attract immune cells including monocytes is a criterion for pro-inflammatory potential of (virus-infected) cells. Migration assays using supernatants from NAC-treated or -untreated uninfected or H5N1-infected (MOI 0.01) A549 cells to attract monocytes were performed. The number of migrated monocytes was five times higher towards supernatants of H5N1-infected cells than towards supernatants of uninfected cells (Fig. 5). NAC 15 mM treatment reduced monocyte migration towards supernatants of infected cells (3.1-fold decrease relative to virus control) (Fig. 5) but did not significantly affect migration of monocytes towards supernatants of mock-infected cells (data not shown).

3.5. Influence of NAC on activation of redox sensitive cellular signalling pathways relevant in influenza pathology

NF-κB and p38 are commonly activated in response to oxidative stress and known to be of relevance for influenza A virus replication and pathology [27–29]. NAC was shown to inhibit both H5N1-induced p38 and NF-κB activation (Fig. 6). NAC 15 mM inhibited H5N1-induced p38 phosphorylation as indicated by Western blot (Fig. 6A). Detection of induction of the NF-κB subunits Rel A (p65) and NF-κB1 (p50) in A549 nuclear cell extracts using a subunit specific NF-κB binding ELISA kit revealed that NAC inhibited H5N1-induced NF-κB activation (Fig. 6B). In untreated H5N1-infected A549 cells nuclear levels of p65 and p50 resulted in relative luminescence units (RLU) of $3.8 \times 10^6 \pm 1.1 \times 10^5$ and $6.7 \times 10^6 \pm 1.9 \times 10^5$ RLU, respectively. In NAC 15 mM-treated H5N1-infected cells nuclear levels of p65 and p50 correlated to $2.4 \times 10^6 \pm 1.6 \times 10^5$ and $4.1 \times 10^6 \pm 4.4 \times 10^5$ RLU, respectively.

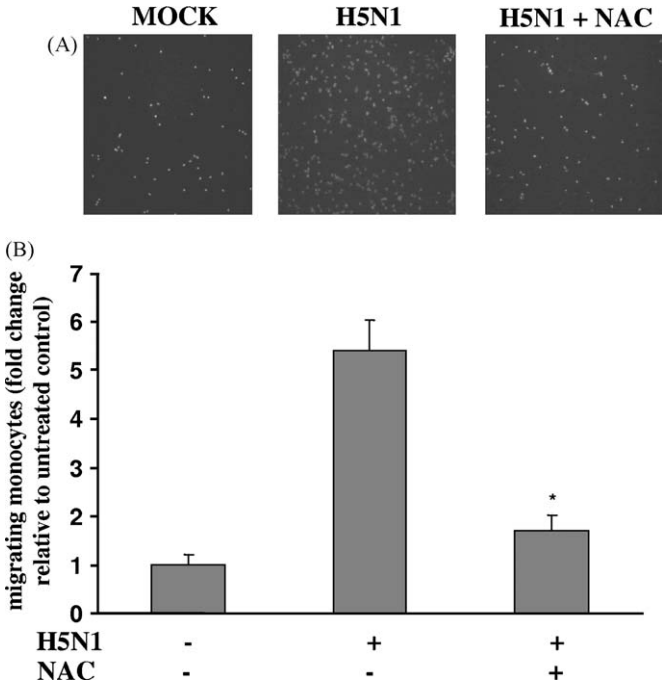

Fig. 5. Influence of N-acetyl-L-cysteine (NAC) treatment on H5N1-induced chemoattraction of monocytes. Motility assays using supernatants (24 h p.i.) from NAC-treated (15 mM, continuously treated starting 24 h prior to infection) or -untreated MOCK- or H5N1-infected (strain A/Thailand/1(Kan-1)/04; MOI 0.01) A549 cells were performed. (a) Representative photographs show monocytes that migrated through 8 μM-filters towards cell culture supernatants. (b) The number of migrated monocytes was determined as fold change relative to untreated mock. Data represent the mean ± SD of three separate experiments. $^*P < 0.05$ relative to untreated virus control.

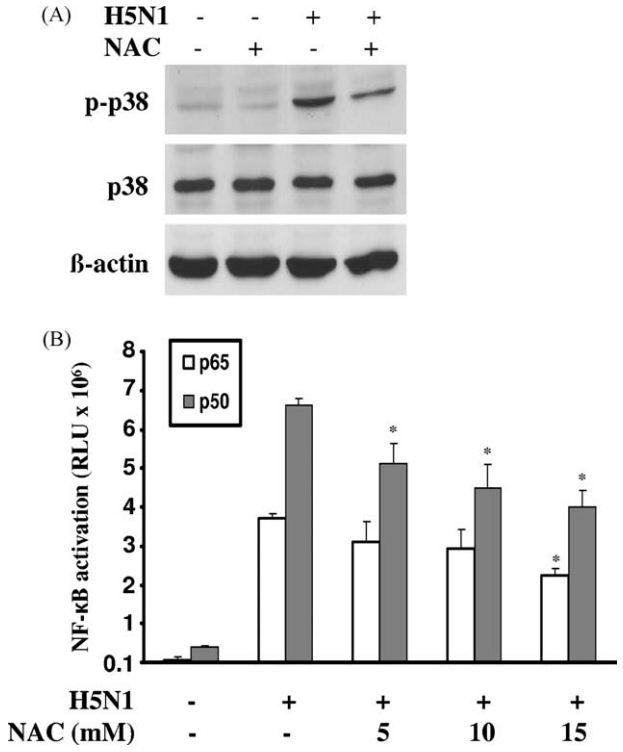

Fig. 6. Influence of N-acetyl-L-cysteine (NAC) on p38 or nuclear factor κB (NF-κB) signalling in H5N1-infected A549 cells. (a) Representative Western blot showing the influence of NAC (15 mM) on the levels of p38 and phosphorylated p38 (p-p38) in H5N1 strain A/Thailand/1(Kan-1)/04-infected A549 cells (MOI 0.01) 24 h post-infection. NAC treatment was performed continuously starting 24 h prior to infection. β-Actin was used as loading control. (b) Effect of NAC on the H5N1-induced induction of the NF-κB subunits Rel A (p65) and NF-κB1 (p50) in A549 nuclear cell extracts using a subunit specific NF-κB binding ELISA kit 24 h post-infection. NAC treatment was performed continuously starting 24 h prior to infection. Data represent the mean ± SD of two separate experiments. $^{*}P < 0.05$ relative to untreated virus control.

3.6. Influence of specific pharmacological inhibitors of p38 or NF-κB on H5N1 replication in A549 cells

To test whether the antiviral activity of NAC may be mediated by inhibition of p38 and/or NF-κB activation, cells were treated with non-toxic concentrations of the p38 inhibitor SB203580 (20 μM) and/or the NF-κB inhibitor BAY 11-7085 (20 μM). Both inhibitors significantly reduced viral titres (SB203580: 15.2-fold decrease relative to virus control; BAY 11-7085: 2.4-fold decrease relative to virus control) (Fig. 7A). The combination of SB203580 and BAY 11-7085 resulted in a further (non-significant, compared to SB203580 treatment) decrease of viral titres (33.2-fold decrease relative to virus control).

3.7. Influence of specific pharmacological inhibitors of p38 or NF-κB on H5N1-induced expression of pro-inflammatory genes in A549 cells

To investigate whether NAC-mediated inhibition of cytokines/chemokines may be mediated by inhibition of p38 and/or NF-κB activation, cells were treated with the p38 inhibitor SB203580 (20 μM) and/or the NF-κB inhibitor BAY 11-7085 (20 μM) and pro-inflammatory cytokines/chemokines were measured by ELISA. Both inhibitors significantly reduced expression of CCL5 (untreated control: 412.6 ± 10.0 pg/ml; SB203580: 125.5 ± 6.3 pg/ml; BAY 11-7085: 246.5 ± 4.4 pg/ml), CXCL8 (untreated control: 989.2 ± 14.8 pg/ml; SB203580: 417.1 ± 12.4 pg/ml; BAY 11-7085: 839.0 ± 10.7 pg/ml), CXCL10 (untreated control: 135.9 ± 12.0 pg/ml; SB203580: 37.2 ± 3.4 pg/ml; BAY 11-7085: 51.7 ± 3.6 pg/ml) and IL-6 (untreated control: 263.6 ± 33.5 pg/ml; SB203580: 13.9 ± 9.3 pg/ml; BAY 11-7085: 115.5 ± 19.8 pg/ml) (Fig. 7B). The combination of SB203580 and BAY 11-7085 resulted in a further significant decrease of cytokine/chemokine expression (CCL5 52.5 ± 16.3 pg/ml; CXCL8: 309.4 ± 26.2 pg/ml; CXCL10: 19.4 ± 3.5 pg/ml; IL-6: concentration below detection limit).

4. Discussion

In the present paper, we demonstrate that NAC treatment inhibited H5N1 influenza A virus replication and H5N1-induced cell death. Moreover, NAC diminished H5N1-induced expression of the cytokines/chemokines CCL5, CXCL8, CXCL10 and IL-6 and migration of monocytes towards supernatants of H5N1-infected cells. NAC is an effective antioxidant. It enriches the intracellular sulfhydryl pool, acting as a precursor of reduced glutathione (GSH) [20]. Protective activity of NAC against seasonal influenza A infection was shown in animal studies by decreasing mortality of mice infected with the influenza A strain A/PR/8 (H1N1) [30]. In humans, NAC significantly reduced the incidence of clinically apparent A/H1N1 disease [20]. In cell culture experiments NAC prevented influenza A (H3N2) virus-induced oxidative stress, cell death, expression of inflammatory genes and NF-κB activity [16,31].

It is probable that NAC antiviral activity against H5N1 virus results from its ability to inhibit activation of intracellular signalling molecules and transcription factors which are sensitive to oxidants produced during influenza A infection. In concordance, NAC treatment reduced formation of ROS in H5N1-infected cells [Figure S1, available as Supplementary data] as well as prevented activation of two constituents of redox sensitive signalling pathways: (1) transcription factor NF-κB and (2) MAPK p38. Both signalling pathways are known to be involved in influenza A virus pathogenesis [28,29,32]. Numerous substances that cause inhibition of NF-κB including the radical scavenger pyrrolidine dithiocarbamate (PDTC), the proteasome inhibitor MG132, the cyclooxygenase (COX) inhibitor acetylsalicylic acid and the specific NF-κB inhibitor BAY 11-7085 were shown to inhibit influenza A virus replication [33–35]. Similar to other NF-κB inhibitors, NAC concentrations required to block viral replication directly correlated with the concentration needed to inhibit NF-κB. Moreover, the NF-κB inhibitor BAY 11-7085 was also able to inhibit H5N1 replication in our setting.

NF-κB activation may result in inhibition or promotion of apoptosis depending on the cell type and the context [36]. NAC had already been shown to increase apoptosis in hypoxic cells through inhibition of NF-κB [37]. However, NAC had previously also been demonstrated to concomitantly impair influenza A (H3N2) virus-induced NF-κB activity and cell death [16]. Moreover, recent results demonstrated that different NF-κB inhibitors suppress apoptosis in H5N1-infected A549 cells [33]. Therefore, inhibition of H5N1 replication as well as inhibition of H5N1-induced apoptosis by NAC in A549 cells may depend on its ability to interfere with virus-induced NF-κB activation. The two major apoptosis signalling pathways are death receptor-induced (extrinsic) apoptosis in which caspase-8 functions as initiator caspase and mitochondrial (intrinsic) apoptosis with caspase-9 as initiator caspase [38]. In our experiments, activation of both initiator caspases-8 and -9 was observed after H5N1 infection suggesting that both apoptosis pathways may be involved in H5N1-induced apoptosis. Since NAC inhibited activation of both initiator caspases it may interfere with both apoptosis pathways. Moreover, inhibition of caspase-3 activity by NAC may be directly relevant for its antiviral effects. Previous studies demonstrated that inhibition of caspase-3 in H5N1-infected A549 cells by acetylsalicylic acid resulted in efficient retention of influenza RNP complexes in the nuclei of infected A549 cells and in turn in inhibition of virus replication

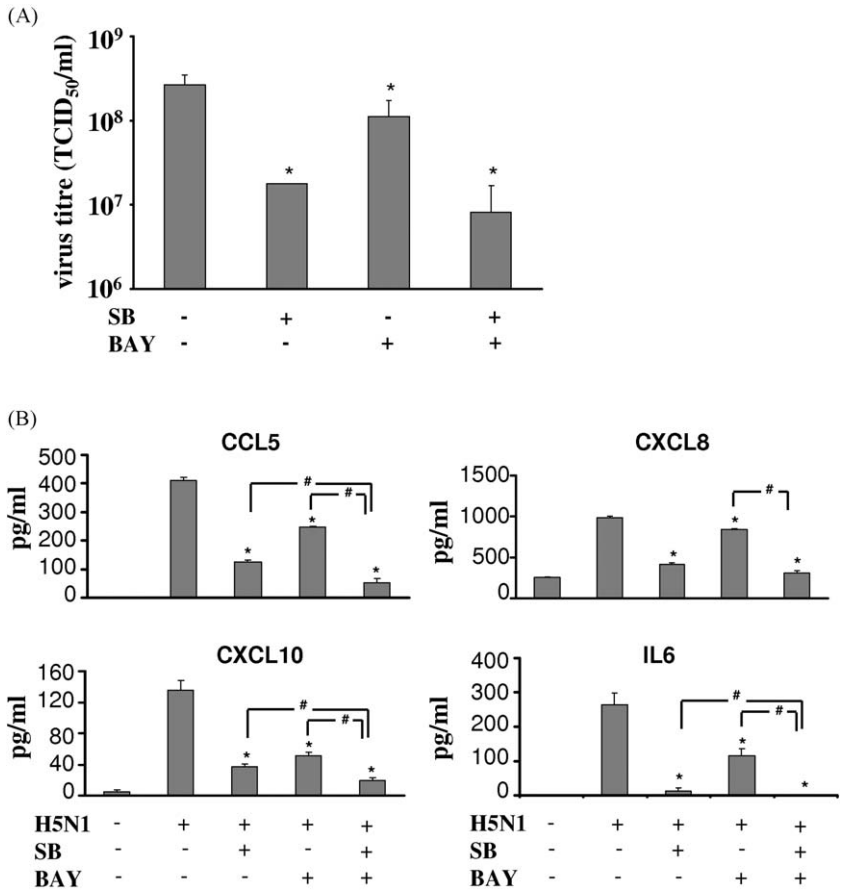

Fig. 7. Influence of specific inhibitors of p38 (SB203580) or NF-κB (BAY 11-7085) on H5N1 replication and cytokine/chemokine expression in H5N1-infected A549 cells. A549 cells were infected with A/Thailand/1(Kan-1)/04 (H5N1) at a MOI of 0.01. Treatment with SB203580 (20 μM) and/or BAY 11-7085 (20 μM) was performed continuously starting 1 h prior to infection. (a) H5N1 titres were determined 24 h post-infection. (b) Expression of CCL5, CXCL8, CXCL10 or IL-6 was determined 24 h post-infection using ELISA. Data represent the mean ± SD of three separate experiments. $^{*}P < 0.05$ relative to untreated virus control. $^{\#}P < 0.05$ relative to SB203580 or BAY 11-7085 treated virus-infected cells.

[33]. In concordance, suppression of caspase-3 activity by NAC was associated with increased retention of RNP in the nuclei of H5N1-infected A549 cells and concomitant inhibition of virus production.

In addition to effects on NF-κB, NAC could influence virus replication by its interference with p38. Influenza A virus infection of different cell types may result in the activation of several MAPKs including p38. Our results also demonstrate that H5N1 infection activates p38 in A549 cells. p38 inhibition did not influence virus replication in macrophages or chorion cells infected with H1N1 or H5N1 virus strains in some previous reports [28,34,39,40]. In MDCK cells, however, the pharmacological p38 inhibitor SB203580 was shown to inhibit RNP transport from the nucleus to the cytosol [32]. Since nuclear export of the RNP complex has been shown to be a critical step in influenza A virus replication this suggests that p38 inhibition may affect H5N1 replication in certain cell types [33]. In our model, the p38 inhibitor SB203580 inhibited H5N1 replication indicating that p38 activation may play a role for H5N1 replication in epithelial cells. The combination of the NF-κB inhibitor BAY 11-7085 and the p38 inhibitor SB203580 caused increased inhibition of H5N1 replication compared to either single treatment. Although differences did not achieve statistical significance this further supports that NAC-induced inhibition of both pathways may contribute to the anti-H5N1 effects exerted by NAC. Notably, NAC had been reported to impair RANTES expression without affecting p38 phosphorylation in H1N1-infected bronchial epithelial cells (cell line NCI-H292) [31]. These varying findings further stress that drug effects may depend on the context and differ between different cells as well as between different viruses.

The mechanism of NAC-induced suppression of chemokine/cytokine production in H5N1-infected cells appears to also involve inhibition of activation of p38 and NF-κB. The p38 inhibitor SB203580 as well as the NF-κB inhibitor BAY 11-7085 both reduced production of pro-inflammatory cytokines/chemokines in H5N1-infected cells. Combination of both substances resulted in a significantly increased suppression of production of pro-inflammatory molecules. Notably, NAC also inhibited H5N1-induced caspase-3 activation [Figure S2, available as Supplementary data] and expression of pro-inflammatory molecules [Figure S3, available as Supplementary data] in human monocyte-derived macrophages indicating that anti-H5N1 effects of NAC may not be limited to epithelial cells.

Both pathways have already been suggested to be involved in H5N1-induced pro-inflammatory signalling. In human primary macrophages, H5N1 and H1N1 viruses did not differ in the activation of NF-κB but unlike H1N1 virus, H5N1 viruses strongly activated p38 resulting in production of inflammatory cytokines and chemokines [28,39]. Similarly to these experiments with human macrophages, the treatment of A549 cells with SB203580 specific p38 inhibitor described here significantly suppressed expression of inflammatory cytokines/chemokines. In a transgenic mouse model with a deletion of p50 NF-κB subunit, H5N1 virus infection resulted in a lack of hypercytokinemia [41]. However, H5N1 pathogenesis was not altered in this model [41]. Moreover, cytokine and chemokine knockout mice or steroid-treated wild-type mice did not have survival advantage over wild-type mice after viral challenge [42]. These data indicate that suppression of

cytokine/chemokine expression alone is not sufficient to improve disease outcome. However, control of excessive inflammation may have beneficial effects in combination with antiviral treatment that reduces virus loads. Indeed, delayed antiviral treatment with neuraminidase inhibitors in combination with immunomodulatory substances reduced mortality in mice infected by high inoculums of H5N1 virus [43]. In these experiments, significant improvements in survival rate, survival time, and inflammatory markers were reported for mice treated with a triple therapy containing zanamivir and immunomodulators including celecoxib, and mesalazine in comparison to zanamivir alone. Zanamivir with or without immunomodulators reduced viral load to a similar extent [43]. Therefore, antioxidants like NAC may serve as additional therapeutic options affecting in parallel H5N1 replication as well as H5N1-induced expression of pro-inflammatory molecules. Although NAC concentrations which showed antiviral and anti-inflammatory effects in H5N1-infected cells are unlikely to be achieved in humans by oral administration, NAC administered by alternative routes (inhalation, parental) may result in therapeutically effective concentrations.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bcp.2009.08.025.

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