Inhibition of Na⁺/H⁺ Exchanger Activity by Parvovirus B19 Protein NS1

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Key Words

Apoptosis • Programmed cell death • NHE • Retinoblastoma cells • Caspases • Tyrosine kinases

Abstract

Infection with parvovirus B19 (B19) may induce apoptosis resulting in anemia, acute fulminant liver failure, placental insufficiency and myocarditis. Apoptosis has been attributed to proapoptotic activity of the non-structural viral protein NS1, which is known to trigger a signaling cascade eventually leading to activation of caspases. In several cell types apoptosis was found to be paralleled by profound cytosolic acidification, which may be secondary to inhibition of the Na⁺/H⁺ exchanger. The acidification has been considered to support the activation of pH sensitive caspases and endonucleases. However, nothing is known about the effect of NS1 on Na⁺/H⁺ exchanger activity and cytosolic pH. The present study thus explored whether NS1 expression affects cytosolic pH (pHi) and Na⁺-dependent realkalinization (Δ pHi) following acidification by an ammonium pulse. According to FACS analysis, overexpression of NS1 in RXR-10SW cells led within 72 hours to activation of caspase 3 and DNA fragmentation. NS1

overexpression resulted within 24 hours in a significant decline of pHi from 6.93 ± 0.03 (n = 6) to 6.78 ± 0.04 (n = 7), and to a significant decrease of Δ pHi from 0.159 ± 0.017 (n = 6) to 0.039 ± 0.004 , (n = 7). The decrease of pHi and of Δ pHi following NS1 expression could be significantly blunted by inhibition of caspase 3 with zVAD. Western blot analysis revealed degradation of NHE1 following NS1 expression. *In vitro*, caspase 3, but not caspase 6, caspase 7 and caspase 8 degraded NHE1 protein of cell lysates. In conclusion, overexpression of NS1 triggers a signaling cascade eventually leading to activation of caspase 3 and subsequent degradation of NHE1. The effect contributes to cytosolic acidification which may in turn favor activation of caspases and endonucleases and thus participate in the pathophysiology of B19-infection.

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Introduction

Parvovirus B19 infections may trigger apoptosis of infected cells, such as erythroid cells [1-6], hepatocytes [7] and placental trophoblast cells [8], thus eventually leading to anemia [1, 9], acute fulminant hepatic failure [7, 10], or foetal death [8], respectively. The apoptotic

effect of parvovirus infection is mimicked by overexpression of parvoviral protein NS1, which interacts with the transcription factors Sp1/Sp2 [11], leading to upregulation of p53, Bax and Bad and to subsequent activation of caspase 9 and caspase 3 [12-14]. The propaoptotic effect of NS1 is abrogated upon loss of function mutations in the nucleoside triphosphate binding domain of NS1 [10, 13].

The 5.6 kb linear single-stranded DNA genome of B19 contains three large open reading frames (ORFs). The first ORF is located at the 5'-half of the genome and encodes the non-structural protein NS1 with a molecular mass of approx. 71 kDa. The NS1 protein represents site-specific DNA-binding and is associated with transcriptional and helicase activities [15-18]. The NS1 protein acts as a transactivator on cellular (e.g. IL-6) and viral (e.g. HIV) promoters [19]. It has been demonstrated that the cytotoxicity of B19 is closely related to the interference of NS1 with elements of the signaling pathways leading to host cell apoptosis, such as p21/WAF and caspase 3 [12-14].

Cellular events paralleling apoptosis include inhibition of the Na^+/H^+ exchanger [20, 21] leading to profound cytosolic acidosis and participating in apoptotic cell shrinkage [21-23]. The cytosolic acidification may support the activation of enzymes catalyzing DNA degradation [24-28]. Accordingly, cytosolic acidification has been shown to accelerate apoptosis of a variety of cells including BAF-3 cells [29], HL-60 cells [24, 30], CTLL-2 cells [31], MCF-7 cells [32] and MOE-7 cells [33].

Effects of NS1 expression on the Na^+/H^+ exchanger have never been analyzed. Thus, the present paper explored the effect of NS1 expression on cytosolic pH and the Na^+/H^+ exchanger activity in human retinoblastoma cells (RXR-SW). Additional experiments were performed to determine the role of caspases in the regulation of Na^+/H^+ exchanger activity.

Materials and Methods

Solutions

Experiments were performed at 37°C in Ringer solution containing (in mM): 125 NaCl, 5 KCl, 1 CaCl_2 , 1.2 MgSO_4 , 2 NaH_2PO_4 , 5 glucose and 32.2 Hepes. Where indicated, a Na^+ free solution was used composed of (in mM): 125 NMDG, 5 KCl, 1 CaCl_2 , 1.2 MgSO_4 , 2 KH_2PO_4 , 32.2 Hepes, 10 Mannitol and 5 Glucose. To yield a Na^+ -free ammonium chloride solution 10 mM NMDG and mannitol were replaced with 20 mM NH_4Cl . For calibration of cytosolic pH, a high K^+ solution was made up from (in mM): 105 KCl, 1 CaCl_2 , 1.2 MgSO_4 , 32.2 Hepes, 5 mannitol

and 10 μM nigericin. The pH of the solutions was titrated to 7.4 or 7.0 with HCl/NaOH, HCl/NMDG and HCl/KOH, respectively, at 37°C.

Plasmids

B19 DNA was isolated from deparaffinized myocardial tissue of a patient with fatal B19-associated inflammatory cardiomyopathy after proteinase K digestion, phenol/chloroform extraction and ethanol precipitation (Accession number: AY768535 and AF162273) as described previously [34]. For cloning of NS1-plasmids the coding NS1-region was amplified by PCR using a high fidelity polymerase system (Roche, Basel, Switzerland). The following primers were used for cloning of the NS1 coding region into the pIND-vector (Invitrogen, Karlsruhe, Germany): sense primer = B19-NS1-382: 5'-AGC GGG CTT TTT TCC CGC CTT ATG CAA ATG GGC AGC CA-3' and reverse primer = B19-NS1-3573 5'-TAA ATA TCT CCA TGG GGT TG-3'. The NS1 amplicons were digested with the restriction enzymes *Sma*I und *Sac*II, and inserted into the pIND vector (Invitrogen, Karlsruhe, Germany). The resulting plasmid pIND-NS1 was analyzed by automated DNA sequencing in both directions (BigDye Terminator Cycle Sequencing, PE Applied Biosystems, Foster City, CA, USA) to verify correct sequences.

Cell Culture

In a first series, stable, inducible B19 NS1-10 SW cell lines were generated by transfection of the pIND-NS1 plasmid in human retinoblastoma cells (10SW) stably expressing the heterodimer ecdysone-retinoid X receptor for binding a modified ecdysone response element in the presence of the ligand Ponasterone A (Invitrogen, Karlsruhe, Germany). Stable NS1-10 SW cells were maintained in an atmosphere containing 5% CO_2 at 37°C in Dulbecco's modified Eagle's Medium supplemented with 10% (v/v) heat-inactivated foetal calf serum, 1% non-essential amino acids, 1% L-glutamine, 5 mM glucose, 100 units/ml penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin, 400 $\mu\text{g}/\text{ml}$ Zeocin (Invitrogen) and 600 $\mu\text{g}/\text{ml}$ G418 (Calbiochem). All cell culture reagents were purchased from Invitrogen (Karlsruhe, Germany). Cells were allowed to recover for 16 hours after plating before addition of ponasterone A (5 μM) to induce B19 NS1 expression. Cells were seeded in 6-well tissue culture plates at 5 x 10⁵ cells per well.

In a second series cells of the immortalized human microvascular endothelial cell line-1 (hMEC-1), kindly supplied by Thomas Wieder, were grown in Dulbecco's modified Eagle's Medium supplemented with 10% (v/v) heat-inactivated foetal calf serum, 1% non-essential amino acids, 1% L-glutamine, 5 mM glucose, 100 units/ml penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin, and 10 ng/mL epidermal growth factor (ICN, Costa Mesa, CA, USA). hMEC-1 cells were seeded in 60 mm cell culture dishes at a concentration of 8 x 10⁵ cells/dish. hMEC-1 cells were stable transfected with the NS1 plasmid and a doxycycline sensitive promotor. To induce NS1 expression, cultivated stable transfected hMEC cells were exposed to 1 $\mu\text{g}/\text{ml}$ doxycycline in medium. To check for an effect of doxycycline, nontransfected hMEC were similarly exposed to doxycycline. As a negative control, stable transfected cells were incubated

in medium without doxycycline. Incubation of hMEC cells in serum-free medium served as a positive control for apoptosis.

Immunofluorescence and Western Blot experiments

Immunofluorescence experiments were performed as described previously [35]. B19 NS1 antigen was detected with monoclonal mouse anti-NS1 antibodies (kindly provided by Dr. Sugamura, Tohoku University) and anti-mouse FITC-conjugated secondary antibodies (Sigma, Taufkirchen, Germany). Nuclei were stained with 1 mg/ml 4,6-diamidino-2-phenylindole-dihydrochloride (DAPI; Roche Diagnostics, Mannheim, Germany). Cells were analyzed using an immunofluorescence microscope (Zeiss; Oberkochen, Germany). Western blot analysis was performed with whole-cell extracts derived from stable B19-NS1-10 SW cell lines after induction with ponasterone A as described previously [35]. B19 NS1 specific signals were detected using mouse monoclonal anti-NS1 antibodies, secondary anti-mouse HRP-FITC antibodies (Dianova, Hamburg, Germany) and the ECL-detection kit according to the manufacturer's instructions (Applied Biosystems, Darmstadt, Germany).

Determination of cytosolic pH

For pH_i measurements cells were incubated in a HEPES-buffered Ringer solution containing 10 μ M BCECF-AM (Molecular Probes, Leiden, The Netherlands) for 10 min at 37°C after induction of NS1 expression with ponasterone A. After loading, the chamber was flushed for 5 min with Ringer solution to remove any deesterified dye sticking to the outside of the cells. The perfusion chamber was mounted on the stage of an inverted phase-contrast microscope (Axiovert 100, Zeiss, Oberkochen, Germany), which was used in the epifluorescence mode with a 40 x oil immersion objective (Fluar 40x/1.30 oil, Zeiss, Oberkochen, Germany). BCECF was successively excited at 490/10 and 440/10 nm, and the resultant fluorescent signal was monitored at 535/10 nm using an intensified charge-coupled device camera (Proxitronic, Bensheim, Germany) and specialized computer software Axon Imaging Workbench (Axon Instruments, Foster City, USA). Intensity ratio data (490/440) were converted into pH values using the high- K^+ /nigericin calibration technique [36].

The solutions, flow lines and perfusion chamber were maintained at 37°C by a thermostatically controlled heating system. The volume of the perfusion chamber was 600 μ l and the flow rate was 4 ml/min for all solutions. For acid loading, cells were transiently exposed to a solution containing 20 mM NH_4Cl leading to marked initial alkalization of cytosolic pH (pH_i) due to entry of NH_3 and binding of H^+ to form NH_4^+ [37]. The acidification of cytosolic pH upon removal of ammonia allowed to calculate the mean intrinsic buffering power of the cells [37], assuming that NH_4^+ and NH_3 are in equilibrium in cytosolic and extracellular fluid and that ammonia leaves the cells as NH_3 :

$$\beta = \Delta[NH_4^+]/\Delta pH_i,$$

where ΔpH_i is the decrease of cytosolic pH (pH_i) following ammonia removal and $\Delta[NH_4^+]$ the decrease of cytosolic NH_4^+ concentration, which is identical to the concentration of NH_4^+ immediately before the removal of ammonia. Given the pK for NH_4^+/NH_3 of 8.9 [38], an extracellular

pH (pH_o) of 7.4 and the NH_4^+ concentration in extracellular fluid ($[NH_4^+]_o$) of 19.37 [20/(1+10 ^{pH_o-pK})]: $[NH_4^+]_i = 19.37 \cdot 10^{pH_o-pH_i}$.

Assessment of apoptosis

Stable NS1-10 SW cells were stimulated with ponasterone A, 50 μ M (Sigma, Taufkirchen, Germany) for 72 hours. Cells were trypsinized for detachment. Annexin-V-Fluos apoptosis detection kit (Roche, Basel, Switzerland) was used to detect phosphatidylserine on the outer leaflet of the cell membrane. In brief 2×10^5 cells were harvested and washed twice with annexin incubation buffer (10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl, 5 mM $CaCl_2$). Cell pellets were resuspended in 50 μ l of Annexin-V-Fluos labelling solution (20 μ l Annexin-V-Fluos labelling reagent in 1 ml annexin incubation buffer), incubated for 15 minutes at room temperature and analyzed by flow cytometry (FACS Calibur, BD Biosciences, Heidelberg, Deutschland).

For determination of DNA fragmentation, 5×10^5 cells were fixed with 0.75% formaldehyde for 30 minutes and then incubated with 70% ice cold ethanol for 15 minutes. Cells were then treated with RNase A for 30 minutes at 37°C, washed and resuspended in 200 μ l Propidium Iodide (0.5 μ g/ml, Sigma, Taufkirchen, Germany). The DNA content of the samples was analyzed by flow cytometry (FACS Calibur, BD Biosciences, Heidelberg, Germany).

Caspase-3 activation was determined using the kit from BD biosciences (Heidelberg, Germany) according to the manufacturer's instructions. Briefly 10^6 cells were washed twice with cold PBS, fixed and permeabilized with 'Cytotfix/Cytoperm' solution and then washed twice with 'Perm/ Wash' buffer. Then cells were stained with FITC conjugated anti-active Caspase-3 antibody in 'Perm/ Wash' buffer for 30 minutes and were analyzed by flow cytometry (FACS Calibur, BD Biosciences, Heidelberg, Germany).

To determine the time course of apoptosis, caspase 3 activation of 2 million hMEC cells was measured using ApoALERT Caspase 3 colorimetric assay kit (Clontech, Saint-Germain-en-Laye, France) according to the manufacturer's protocol. Furthermore, apoptosis-related cell membrane scrambling of 3×10^4 cells was determined using the APOPercentage Apoptosis Assay kit (Biocolor, Carrickfergus, UK).

Degradation of NHE1

To explore, whether NS1 expression leads to degradation of NHE1 protein, lysates were generated from stable NS1-10 SW cells without or with prior ponasterone A treatment and NHE1 protein analyzed by Western blotting using a NHE1 specific antibody (BD Biosciences). *In vitro* caspase assays were performed as described previously [39]. Briefly, cytosolic extracts of 2×10^6 Jurkat cells were prepared and preincubated for 3 min in the presence or absence of 200 μ M zVAD-fmk. Subsequently, 2 μ g of purified caspase 3, caspase 6, caspase 7, or caspase-8 were added and incubated in a total volume of 50 μ l at 37°C for 2 h. Reactions were stopped by the addition of 4x SDS-loading buffer, loaded on a SDS polyacrylamide gel, and immunoblotted for NHE1 (BD Biosciences, Heidelberg, Germany).

Fig. 1. Expression of NS1 proteins in stable inducible retinoblastoma cells: A. Immunofluorescence experiments demonstrating NS1 protein expression after induction with ponasterone A (upper panel). NS1 protein was detected with monoclonal anti-NS1 and secondary FITC-labeled goat anti-mouse antibodies 48h after induction. No NS1 protein expression is visible in retinoblastoma cells without ponasterone A stimulation (lower panel). The corresponding images of DAPI staining are shown on the right. B. Western blot analysis demonstrating expression of NS1 polypeptides (71 kDa) from ponasterone A induced NS1 stable retinoblastoma cells (lane 2). NS1 retinoblastoma cell extract not stimulated with ponasterone A (lane 1) and retinoblastoma control cell extracts (lane 3) are shown. B19-specific NS1 polypeptides were detected using monoclonal anti-NS1 antibodies.

Statistics

Data are provided as means $\pm$ SEM, n represents the number of independent experiments. All data were tested for significance using Student t-test or ANOVA (Dunnets test), where applicable, and only results with $P < 0.05$ were considered statistically significant.

Results

Cloning and expression of B19 NS1-protein in stable transfected cell lines

In order to determine the effect of the B19 non structural protein NS1 on the regulation of the Na^+/H^+ exchanger activity and cytosolic pH, we amplified the coding NS1 gene from myocardial tissue of a B19-infected patient by PCR. The NS1 patient-specific PCR-products were introduced into eukaryotic expression vectors (pIND) under the control of an ecdysone-inducible minimal heat shock promoter. The retinoblastoma cell line VgRXR-10SW expressing the heterodimer ecdysone-retinoid X receptor (RXR) that binds the ecdysone-analog ponasterone A was used to generate the stable, inducible B19-protein cell lines NS1-10 SW. Upon addition of ponasterone A the expression of the B19-NS1 protein was induced. In order to determine expression of NS1, RT-PCR (data not shown), immunofluorescence (Fig. 1A) and Western blot experiments (Fig. 1B) were performed. As demonstrated by these experiments the NS1 proteins could be clearly detected in 80% to 90% of NS1-10 SW cells after induction with ponasterone A by immunofluorescence (Fig. 1A) and in correct sizes using Western Blot analyses (Fig. 1B).

Fig. 2. Annexin V binding of retinoblastoma cells following NS1 expression: A. Histograms showing annexin V-binding of retinoblastoma cells transfected with NS1-containing ponasterone A inducible vector prior to (left panels) and following (right panels) stimulation of NS1 expression with ponasterone A. B. Means $\pm$ SEM (n = 3) of the percentage of annexin V-binding retinoblastoma cells transfected with NS1 prior to (open bars) and following (closed bars) stimulation of NS1 expression with ponasterone A.

Fig. 3. DNA fragmentation in retinoblastoma cells following NS1 expression: A. Histograms showing DNA fragmentation of retinoblastoma cells transfected with NS1-containing ponasterone A inducible vector prior to (left panels) and following (right panels) stimulation of NS1 expression with ponasterone A. B. Means $\pm$ SEM ($n = 4$) of the percentage of retinoblastoma cells with fragmented DNA transfected with NS1 prior to (open bars) and following (closed bars) stimulation of NS1 expression with ponasterone A.

Annexin V binding, DNA fragmentation and caspase activation after expression of NS1

Retinoblastoma cells were treated with ponasterone A to induce NS1 protein expression. Expression of NS1 in retinoblastoma cells led to break down of phosphatidylserine asymmetry across the cell membrane which was apparent from annexin V-binding (Fig. 2). Annexin V-binding averaged $4.98 \pm 0.28\%$ ($n = 3$) in retinoblastoma cells transfected with NS1 vector but not treated with ponasterone A. A significantly higher percentage of annexin V-binding ($10.24 \pm 0.75\%$, $n = 3$) was observed in retinoblastoma cells expressing NS1 following ponasterone A stimulation.

The expression of NS1 was further followed by triggering of DNA fragmentation (Fig. 3). Again, significantly more DNA fragmentation was observed in ponasterone A treated retinoblastoma cells transfected with the NS1 vector than in NS1 transfected cells without ponasterone A treatment.

As illustrated in Fig. 4, the annexin V-binding and DNA fragmentation were paralleled by caspase activation. Again, more caspase activity was observed in

Fig. 4. Caspase activation in retinoblastoma cells following NS1 expression: A. Representative dot plots of caspase activation in retinoblastoma cells transfected with NS1-containing ponasterone A inducible vector prior to (left panels) and following (right panels) stimulation of NS1 expression with ponasterone A. Numbers depict the percentage of cells with activated caspases. B. Means $\pm$ SEM ($n = 3$) of the percentage of retinoblastoma cells with active caspase 3 transfected with NS1 prior to (open bars) and following (closed bars) stimulation of NS1 expression with ponasterone A.

ponasterone A treated retinoblastoma cells transfected with NS1 than in NS1 transfected cells not treated with ponasterone A.

Further experiments were performed to investigate the time course of NS1-dependent stimulation of apoptosis and stable transfected hMEC cells. To this end, the expression of NS1 was started by addition of doxycycline to the cell culture medium. Then, caspase 3 activation and cell scrambling were assessed after 24, 48, and 72 hours of incubation. As shown in Fig. 5A, after 24 hours, caspase 3 was significantly activated in doxycycline-treated stable transfected cells, while neither a doxycycline treatment of nontransfected hMEC cells nor incubation of non-stimulated transfected cells resulted in appreciable caspase 3 activation. Subsequently, Apoptosome-dependent light absorption was employed as a measure of cell scrambling due to apoptosis. As shown in Fig. 5B, a 24 hours doxycycline treatment significantly induced apoptosis in stable transfected hMEC cells, an effect, which was even more pronounced after 48 and 72 hours.

Fig. 5. Time course of NS1-dependent caspase activation and membrane scrambling in stable transfected hMEC cells: A: Time course of caspase 3 activation of NS1-stable transfected hMEC cells incubated in the absence (non-induced) or presence (induced) of doxycycline or of non-transfected hMEC cells incubated in the presence of doxycycline (doxycycline without vector). As a positive control, hMEC cells incubated in the absence of serum were analysed. Arithmetic means $\pm$ SEM ($n = 4$) of caspase 3 activation, calculated from the absorption at 405 nm by use of a standard curve. B: Time course of apoptosis-dependent cell membrane scrambling of NS1-stable transfected hMEC cells incubated in the absence (non-induced) or presence (induced) of doxycycline or of non-transfected hMEC cells incubated in the presence of doxycycline (doxycycline without vector) or of hMEC cells incubated in the absence of serum (positive control). Arithmetic means $\pm$ SEM ($n = 4$) of caspase 3 activation-dependent absorption at 550 nm.

Fig. 6. Effect of NS1 expression on cytosolic pH of retinoblastoma cells: Cytosolic pH prior to, during and following an ammonium pulse. To load the cells with H^+ , 20 mM NH_4Cl was added and Na^+ removed (replaced by NMDG) in a first step (NH_4Cl), NH_4Cl removed in a second step (0 Na^+), and Na^+ added (Na^+) in a third step. A. Original tracings illustrating alterations of pH in typical experiments in retinoblastoma cells transfected with NS1-containing ponasterone A inducible vector prior to (upper panels) and following (lower panels) stimulation of NS1 expression with ponasterone A. B. Arithmetic means $\pm$ SEM ($n = 7$) of pH_i in retinoblastoma cells transfected with NS1-containing ponasterone A inducible vector before (open bars) and after (closed bars) stimulation of NS1 expression with ponasterone A treatment. *indicates statistically significant difference ($p < 0.05$) between cells transfected with NS1-containing ponasterone A inducible vector in presence or absence of ponasterone A. C. Arithmetic means ($\pm$ SEM; $n = 7$) of the increase in pH_i ($\Delta pH/min$) in retinoblastoma cells transfected with NS1-containing ponasterone A inducible vector prior to (open bars) and following (closed bars) stimulation of NS1 expression with ponasterone A. # indicates significant difference ($P = 0.05$, ANOVA) to respective value.

Cytosolic acidification in NS1 expressing retinoblastoma cells

Expression of NS1 in retinoblastoma cells was followed by marked cytosolic acidification (Fig. 6). Initial cytosolic pH in retinoblastoma cells, transfected with a NS1-containing ponasterone A inducible vector, was significantly lower following ponasterone A treatment (6.78 ± 0.04 , $n = 7$) than in the absence of ponasterone A (6.93 ± 0.03 , $n = 6$).

Ammonium pulses were utilized to load the cells with H^+ [37]. The superfusion with 20 mM NH_4Cl and simultaneous removal of Na^+ (replacement with NMDG $^+$) was followed by alkalinization (Fig. 6). The subsequent

replacement of NH_4Cl with NMDG Cl $^-$ in the continued absence of Na^+ led to a sharp acidification due to exit of NH_3 and retention of H^+ within the cells. The acidification allowed the calculation of an apparent buffer capacity (see methods) which was not significantly different between NS1 expressing retinoblastoma cells following treatment with ponasterone A (71 ± 11 mM/pH, $n = 7$) and transfected retinoblastoma cells without ponasterone A treatment (49 ± 11 mM/pH, $n = 6$).

The acidification was followed by a pH recovery, which was markedly accelerated by subsequent replacement of NMDG with Na^+ (Fig. 6). The Na^+ -dependent realkalinization (ΔpH_{Na}) was significantly

blunted in NS1 expressing retinoblastoma cells (Fig.6).

As illustrated in Fig. 7, the inhibitory effect of NS1 on the Na^+/H^+ exchanger was prevented in the presence of caspase inhibitor z-VAD-fmk (20 μM). In NS1 expressing cells the cytosolic pH was significantly higher in the presence than in the absence of caspase inhibitor zVAD. Moreover, the treatment of the cells with zVAD abrogated the inhibitory effect of NS1 expression on the Na^+ - dependent cellular realkalinization following an ammonium pulse. Thus, the inhibitory effect of NS1 on NHE1 requires activation of caspases.

Caspase 3 dependent degradation of NHE1 protein due to NS1 expression

The inhibition of NHE1 activity following NS1 expression could at least partially have been due to caspase dependent degradation of the NHE1 protein. To explore this possibility, Western blots were performed utilizing NHE1 specific antibody staining. As shown in Fig. 8A and B, expression of NS1 was indeed followed by significantly higher levels of degraded NHE1 protein in Western blots of cell lysates. Treatment of cell lysates with caspases revealed that caspase 3, but not caspase 6, caspase 7 and caspase 8, is capable to degrade NHE1 protein (Fig. 8C).

Discussion

Previous reports have implicated B19 infection in proapoptotic processes due to overexpression of NS1 proteins [10, 13, 40]. Persistence of B19 genomes in various tissues has been reported, however, the significance of the etiopathogenetic relevance of B19 persistence is still under discussion [41-44]. Restricted replication of B19 in different cell types has been shown previously which may be due to aberrant splicing and transcription of B19 capsid proteins VP1 and VP2, whereas the non structural protein NS1 is not affected [45, 46]. In order to determine possible pathophysiological mechanisms of NS1 proteins we have generated stable inducible NS1 expressing retinoblastoma cell lines.

The present experiments disclose the strong impact of parvoviral non structural protein NS1 on cytosolic pH regulation. We demonstrate that expression of NS1 leads to inhibition of Na^+/H^+ exchanger activity resulting in profound acidification of the B19 NS1 expressing cells. In the absence of Na^+ , the realkalinization is slow, indicating that the Na^+/H^+ -exchanger is the major pH regulator under those experimental conditions. The experiments were performed in the absence of HCO_3^- to avoid pH regulation by HCO_3^- transport.

Fig. 8. Degradation of NHE1 by NS1 expression and *in vitro* incubation with caspase 3: A: Original Western blots demonstrating NHE1 antibody staining of lysates from retinoblastoma cells transfected with either empty (control) or NS1-containing (NS1) vector. B: Arithmetic means $\pm$ SEM (n = 4) of cells transfected with an empty vector (open bar) or cells transfected with NS1 and incubated for 72 hours (closed bar). * indicates statistically significant difference (p<0.05) between cells transfected with empty vector and cells transfected with NS1. C: Cytosolic extracts of 2 x 10⁶ Jurkat cells were preincubated in the presence or absence of 200 μ M zVAD-fmk and subsequently incubated with purified caspase 3, caspase 6, caspase 7, or caspase-8 at 37°C for 2 h. Filled arrowheads indicate the uncleaved 92 kDa full-length form and open arrowheads the cleaved p57 form of NHE1. Asterisk indicates a non-specific band.

The present observations further reveal that NS1 associated apoptosis is paralleled by degradation of NHE1. As DNA fragmentation precludes de novo synthesis of NHE1 protein, the effect of the caspases leads to irreversible elimination of Na⁺/H⁺ exchanger activity. Even though we cannot exclude the contribution of further NHE isoforms to Na⁺-dependent pH recovery, degradation of NHE1 presumably contributes to the inhibition of Na⁺/H⁺ exchanger activity.

The acidifying effect of NS1 expression is paralleled by activation of caspases, key effectors of apoptotic death of a wide variety of cells [7, 47-63] including cardiomyocytes [64, 65]. Caspase inhibition blunts cytosolic acidification or inhibition of Na⁺/H⁺ exchanger activity. Earlier studies revealed that zVAD-fmk did not interfere with the cytosolic acidification following interleukin-3 withdrawal [29] or following staurosporine exposure [66]. However, in contrast to mitochondrial stimuli of apoptosis, death receptor induced intracellular acidification is apparently mediated via caspases [67]. It should be kept in mind, though, that cytosolic acidification may result from further cellular mechanisms, i.e. the activation of HCO₃⁻ permeable anion channels [23, 68], Cl⁻/HCO₃⁻ exchangers parallel to Cl⁻ channels [69], and enhanced mitochondrial H⁺ release [66].

Caspase activity and DNA fragmentation may in turn be enhanced by cytosolic acidification. Previous observations disclosed that the type-II DNase has a pH optimum at pH 5.0 [24-28]. Another type of endonuclease has a pH optimum at 7.5 [70], which may provide an explanation for the paradoxically enhanced apoptosis in some cells following cytosolic alkalinization [71, 72].

The pH sensitivity of caspase activity and the caspase dependent degradation of the Na⁺/H⁺ exchanger may establish a positive feedback loop eventually leading to irreversible destruction of the main cellular pH regulator.

While NS1 expression led to a quantitatively variable degree of acidification, apoptosis was detected in only a fraction of cells expressing NS1. The execution of apoptosis depends on the balance of proapoptotic and antiapoptotic mechanisms and may not invariably follow NS1 expression. On the other hand, in most cells, caspase activation was apparently sufficient to induce cytosolic acidification. It should be kept in mind, though, that the pH measurements were performed in the absence of serum and under experimental conditions not identical to those followed by the determination of apoptosis.

In conclusion, expression of the parvoviral protein NS1 leads to profound cytosolic acidification, which is at least in part due to caspase dependent degradation of the Na⁺/H⁺ exchanger NHE1. The effect contributes to the proapoptotic effect of infection with parvovirus B19 and may thus participate in the generation of organ failure. On the other hand, apoptosis of infected cells may trigger phagocytosis and lysosomal degradation and thus clearance of the infected cell along with the virus.

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