Inhibition of the Na⁺-H⁺ Exchanger Isoform-1 and the Extracellular Signal-Regulated Kinase Induces Apoptosis: a Time Course of Events

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Key Words

NHE1 (Na⁺/H⁺ Exchanger Isoform 1) • ERK (Extracellular signal-Regulated Kinase) • HEp-2 Cell Line • Apoptosis

Abstract

Aims: The present study attempts to shed light on the role and the relative position of the Na⁺/H⁺ exchanger isoform 1 (NHE1) and the extracellular signal-regulated kinase (ERK) in HEp-2 cell signaling pathways concerning a diverse range of cellular functions such as regulation of intracellular pH (pHi), DNA synthesis, production of reactive oxygen species (ROS) and apoptosis. **Methods:** Pharmacological inhibition with cariporide (highly specific inhibitor of NHE1) and PD98059 (specific inhibitor of the upstream activator of ERK) was implemented. Fluorescence spectrometry, atomic absorption spectrometry and ELISA methods were used in order to obtain the results. **Results:** NHE1 and ERK take

part in all of the aforementioned cellular functions, as their inhibition had an effect on all of them. Additionally, inhibition of NHE1 resulted in ERK inhibition as well. Moreover, continuous inhibition of NHE1 or ERK for up to 24h led HEp-2 cells to apoptosis, as assessed through caspase-3 activation, DNA fragmentation and annexin-V binding levels. **Conclusion:** Our data shows a time course of events in relation to NHE1 and ERK and suggests the existence of a positive feedback loop between NHE1 and ERK which could pose a barrier against apoptosis.

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Introduction

The Na⁺/H⁺ exchangers (NHEs) are transmembrane proteins involved in the exchange of extracellular Na⁺ with intracellular H⁺ in a 1:1 ratio in a variety of eukaryotic cells. In mammals 9 isoforms of NHEs have been identified, differing to a varied degree in their amino acid

sequences and their intracellular localization. The Na⁺/H⁺ exchanger isoform-1 (NHE1) is the only ubiquitous member of the family [1] and is a major regulating element of intracellular pH (pHi) [2, 3], cytoskeletal organisation [4, 5], proliferation [6] and cell volume control [7, 8]. Its activity is regulated by a diverse range of factors, such as hormones [9], second messengers [10] and osmotic stress [6]. In previous studies we, amongst others, found that adrenaline [11], glucose [12] and even heavy metals [13, 14] have the ability to modulate NHE1 activity.

ERK belongs to the family of mitogen-activated protein kinases (MAPKs) which play a major role in signaling pathways concerning differentiation, proliferation and survival [15]. Communication between NHE1 and ERK has been suggested [16, 17]. In particular it has been shown that ERK is indirectly involved in growth factor-induced [18], as well as zinc-induced NHE1 activation [13]. Direct involvement of ERK in the alpha(1)-adrenergic activation of NHE1 has also been suggested in a recent study in CCL39 fibroblasts [19]. To the best of our knowledge this is the first time NHE1-ERK interaction in the context of apoptosis has been studied in HEp-2 cells (cell line established via HeLa cell contamination).

Apoptosis is usually a normal, evolutionarily conserved cell procedure, also known as programmed cell death (PCD), which can be observed as a series of events which occur in specific order. It normally plays a very important protective and homeostatic role by removing aged or damaged cells. Two of the main characteristics of apoptotic cells are cytoplasmic acidification and cell volume reduction. Furthermore, cytoplasmic acidification is required in order for pro-apoptotic proteins such as caspases to function properly [20]. On the other hand, cancer cells, as well as all cells undergoing mitosis, exhibit pHi values, which are above normal levels [21]. NHE1 could have an anti-apoptotic role by posing a barrier against cytoplasmic acidification and furthermore by inhibiting cell volume reduction [22]. Studies have linked NHE1 to cell survival. In particular, when NHE1 is activated, apoptosis is inhibited [23]. Recently, it was proposed that NHE1 is a cell survival factor which mediates its role, at least in part, through the ERM (Ezrin/Radixin/Moesin) complex proteins [24]. In contrast to the other members of the MAP kinase family, ERK is generally considered to convey a pro-survival signal [25] in part through activation of transcription factors such as c-fos and elk-1.

In the present study, we attempted to investigate the time course of key cellular procedures such as DNA

synthesis, reactive oxygen species (ROS) production and apoptosis following inhibition of NHE1 and ERK in cells of the HEp-2 cell line. To this end, we used the highly specific NHE1 inhibitor cariporide at three different concentrations. The MAP kinase kinase (MEK1, upstream activator of ERK) inhibitor PD98059 in three different concentrations was also used in order to assess the possible role of ERK in the signaling pathways involving NHE1. In certain experiments only the most potent concentrations of the two inhibitors were used. Our results suggest the existence of a positive feedback loop between NHE1 and ERK, which translates into activation of one from the other, leading to cell survival. Moreover, as observed through the time course results, there are indications that inhibition of either NHE1 or ERK for up to 24h might have the capacity to initiate apoptosis.

Materials and Methods

Materials

PD98059 (2'-Amino-3'-methoxyflavone), nigericin, iodoacetic Na, methazolamide, DIDS (4,4'-diisothiocyanatostilbene-2,2'-disulphonic acid), DCF-DA (2',7'-dichlorodihydrofluorescein diacetate) were from Sigma. BCECF-AM (2',7'-bis(2-carboxyethyl)-5(6)-carboxyfluorescein acetoxymethyl ester) was from AppliChem. [³H] thymidine was from Amersham. Cariporide was from Sanofi-Aventis. Dulbecco's Modified Eagle Medium (DMEM), fetal calf serum (FCS), penicillin/streptomycin, L-glutamine were from Biochrom. All other reagents were of analytical grades and were obtained from commercial sources.

Culture of HEp-2 cells

Cells of the HEp-2 (ATCC® Number: CCL-23™) cell line were used. Cells were cultured in 75 mm flasks at 37°C in a humidified atmosphere of 5% CO₂/95% air. The growth medium was DMEM containing 10% FCS, 2% NaHCO₃ 0.075 g/ml, 100 U/ml penicillin, 100 U/ml streptomycin, 1% L-glutamine and 1% Hepes 1M. After export from the flasks and during the experiments the same growth medium was used.

pHi determination

Changes in pHi were measured through modification of a previously described experimental procedure [26]. In brief, HEp-2 cells suspended in appropriate buffer were loaded with 2 µg/10⁶ cells BCECF-AM for 30 min at 37°C in the dark. BCECF-AM is readily cleaved towards the fluorescent form BCECF by intracellular esterases. Cells were then washed three times with the same buffer in order to remove the fluorescent probe remaining in the buffer. When appropriate, cariporide or PD98059 was added and incubation took place at 37°C in the dark. Along with the inhibitors, 1 mM iodoacetic Na (glycolysis inhibitor), 0.125 mM DIDS (HCO₃⁻/Cl⁻ exchanger inhibitor) and

0.4 mM methazolamide (carbonic anhydrase inhibitor) were added to all samples (including the control) in order to avoid any interference from other pH-regulating systems. Fluorescence was measured immediately after incubation under continuous magnetic stirring at 20°C in a 3 ml quartz cuvette in a Shimadzu fluorescence thermostatic spectrophotometer. Data was obtained as the ratio of the pH-sensitive excitation wavelength (495 nm) to the pH-insensitive excitation signal wavelength (440 nm) with the emission wavelength set at 530 nm. Routinely at each experiment calibration of fluorescence versus pH was conducted through the use of the polyether ionophore nigericin (13 μ M) which couples K⁺ and H⁺ gradients across the plasma membrane, as previously described [27]. The calibration curves produced were roughly linear in the range of pH 6.5-7.5 ($R^2=0.81-1$).

Measurement of Na⁺ uptake

Uptake of Na⁺ was measured as previously described [28]. In brief, washed HEp-2 cells were suspended in Na⁺-free buffer in order to deplete cells of Na⁺. Subsequently, the intracellular Na⁺ concentration was calculated for the cells suspended in a solution of pH 8.0, which represents the total Na⁺ influx, whereas the cell intracellular Na⁺ concentration in a suspension of pH 6.0 represents the influx due to passive permeability alone. For this reason in order to estimate Na⁺ uptake we used two cell samples, each suspended in a buffer with the appropriate pH (6.0 and 8.0). Na⁺ concentration was checked for each couple of samples at three time points. The Na⁺ uptake calculated at each pH was almost linear with time and thus Na⁺ uptake was calculated after a linear regression was conducted. The difference between the two influxes represented the Na⁺ influx stimulated by the pH gradient, and thus is expressed as the influx of Na⁺ due to maximal activity of NHE1. Intracellular Na⁺ concentration after cell lysis was estimated using a Perkin-Elmer atomic absorption spectrophotometer.

ERK activity assay

HEp-2 cells were suspended in a buffer with the following composition (mM): 135 NaCl, 5 KCl, 1 MgCl₂, 1 CaCl₂, 10 glucose, 20 Hepes, pH 7.3. When appropriate cariporide or PD98059 was added to the suspension followed by incubation at 37°C. Samples containing 2x10⁷ cells were taken at 0 and 30 min. The cell suspension was centrifuged, placed on ice and lysed with pre-chilled lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 0.5 mM EDTA, 1 mM DDT, 1% Triton X-100, 0.1% SDS, 100 μ g/ml PMSF, 1 μ g/ml aprotinin, 2 μ g/ml leupeptin, 100 μ g/ml sodium vanadate) by sonication. Cells were then centrifuged at 12 000g for 10 min. ERK activity of clear lysate was then estimated using a commercial assay kit (Chemicon International Inc., Temecula, CA, USA) according to the instructions of the manufacturer. The kit utilized the ELISA principle to detect the phosphorylated form of a specific biotinylated substrate.

Measurement of ERK phosphorylation

The phosphoplas p44/42 MAP kinase (Thr 202/Tyr 204) antibody kit (Cell Signaling technology, Beverly, MA, USA) was used for the Western blot. In brief, HEp-2 cells were immunoblotted with rabbit polyclonal antibody specific to the

phosphorylated form of ERK. Protein extracts were prepared by lysis and separation in 10% SDS-PAGE gels. After electrophoresis the gel was wetted in transfer buffer containing 25 mM Tris, 0.192 M glycine, 10% methanol, 0.01% SDS, pH 8.5 for 15 min and using 2 Whatmanns blotting took place overnight. After the transfer, the membrane was washed with 25 ml TBS 1x for 5 min at room temperature and then incubated in 25 ml blocking buffer containing 1x TBS, 0.1% Tween-20 with 5% non-fat dry milk for 1h at room temperature. Following that, the membrane was washed (3 times for 5 min each) with 15 ml TBS/Tween 1% and then incubated with primary antibody in 10ml antibody solution (1:1000) with gentle agitation overnight at 4°C. After this, the membrane was again washed (3 times for 5 min each) with 15 ml TBS/Tween 1%. Following this, the membrane was incubated with HRP-conjugated secondary antibody (1:1000) and HRP-conjugated antibiotin antibody (1:500) in order to detect biotinylated protein markers in 10 ml blocking buffer, with gentle agitation for 1h at room temperature. The membrane was again washed (3 times for 5 min each) with 15 ml TBS/Tween 1%. Finally, the membrane was incubated with LumiGLO (0.5 ml 20x LumiGLO, 0.5 ml 20x peroxide and 9 ml Milli-Q water) with gentle agitation for 1 min at room temperature and was then wrapped in Saran wrap and exposed to X-ray film (exposure time 10 sec). The markers used were from the soyabean trypsin inhibitor (20.5 kDa), rabbit muscle triosephosphate isomerase (28 kDa), porcine muscle lactate dehydrogenase M (37.5kDa), fused E. coli maltose-binding protein and β -galactosidase (46.5kDa).

Measurement of [³H] thymidine incorporation to DNA

A pulse-labeled technique was used. In brief, approximately 10⁶ HEp-2 cells were placed in each well in a 96-well plate and were incubated for 24 h at 37°C in order for them to adhere to the wells. The supernatant in each well was then removed. Complete medium was added to the wells and cariporide or PD98059 in two thirds of them. The other wells served as a control population. 60 μ l [³H] thymidine (6 μ Ci) were added to every well which was used. Cells were incubated at 37°C. The supernatant was then removed from the wells followed by one wash with 800 μ l PBS. Lysis buffer was added to the wells at 60°C. The supernatant from each well was transferred to a separate tube and measured in a beta scintillation counter.

Measurement of caspase-3 activation

For the measurement of caspase-3 activation we used the "Caspase-3 Colorimetric Activity Assay Kit" (Chemicon International Inc., Temecula, CA, USA). The assay is based on spectrophotometric detection of the chromophore p-nitroaniline (pNA) after cleavage of the caspase-3 specific substrate Ac-DEVD-pNA. HEp-2 cells were incubated with cariporide or PD98059 at 37°C. After centrifugation (1500 rpm) and supernatant abortion, the cells were suspended in chilled lysis buffer, incubated on ice for 10 min and then centrifuged in a microcentrifuge (10 000 g / 5 min). The supernatant (cytosolic extract) from each well was transferred to a fresh tube and put on ice. The same procedure was also undertaken for cells, which constituted the control population. The protein concentrations

Fig. 1. Effect of cariporide (2nM, 20nM, 200nM) on HEp-2 cell intracellular pH. The arithmetic means of at least six experiments are shown. Error bars indicate standard deviations (SDs). The level of significance of the differences between the samples was calculated by ANOVA and Student-Newman-Keuls post-test. $P < 0.05$ for the time points after and including 2 h compared to the control.

of the samples were assayed using the Bradford method in order to quantify cell numbers and to be able to compare results between different experiments. The samples were transferred to wells in a 96-well plate and incubated with 0.03 mg/ml caspase-3 substrate in the dark for 2 h at 37°C. Free pNA was quantified using a microtiter plate reader at 405 nm.

Measurement of DNA fragmentation levels

For the measurement of DNA fragmentation levels we used the “ssDNA Apoptosis ELISA Kit” (Chemicon International Inc., Temecula, CA, USA). The procedure was based on the selective denaturation of DNA in apoptotic cells by formamide and detection of denatured DNA with anti-mouse IgM monoclonal antibody specific to single-stranded DNA (ssDNA). The mAb was conjugated with HRP, thus allowing quantification on a microtiter plate reader at 405 nm. In brief 5000 cells were seeded to wells in a 96-well plate and left to adhere for 24 h at 37°C. Cariporide or PD98059 was added to some of the wells while the rest served as the control population, followed by incubation at 37°C. The supernatant was then removed, 200 µl of fixative were added and the plate was left at room temperature for 30 min. After this the fixative was removed and the plate was incubated in a waterbath at 37°C for 20 min. Cells were incubated with 50 µl of formamide for 10 min at room temperature and then for 10 min at 75°C. The plate was chilled in a refrigerator at 4°C for 5 min followed by supernatant removal. 200 µl of 3% w/v non-fat dry milk were added to each well and incubated for 1 h at 37°C. After supernatant removal, 100 µl of antibody mixture were added to the wells and they were incubated at room temperature for 30 min. There followed three washes with 250 µl of kit wash buffer. Subsequently, 100 µl of ABTS (2,2'-AZINO-bis [3-ethylbenziazoline-6-sulfonic acid]) were added to each well, as well as to another well which served as a blank and incubation took place at 37°C for 45 min. Finally, 100 µl of kit stop solution were added to the wells and DNA fragmentation levels were measured using a microtiter plate reader at 405 nm.

Measurement of annexin-V binding

For the measurement of annexin-V binding to apoptotic HEp-2 cells we used the “Vybrant™ Apoptosis Assay Kit #2” (Molecular Probes Inc., Leiden, the Netherlands). The kit

included annexin-V, which was conjugated with Alexa Fluor® 488 dye. This dye enabled quantification of the percentage of apoptotic HEp-2 cells in a Shimadzu fluorescence spectrophotometer. In brief, cells were incubated in their flask with cariporide or PD98059 at 37°C. An equal amount of cells was also incubated without inhibitors and served as the control population. 5×10^5 cells from each flask were then transferred to eppendorf tubes and centrifuged 3000 rpm / 30 sec. After supernatant removal the cells were washed with chilled 1x PBS. 200 µl of 1x assay buffer was added and cells were incubated with 5 µl Alexa Fluor 488 annexin-V stock solution in the dark at RT for 20 min. The cells were then washed three times with chilled PBS and transferred to larger tubes containing 3 ml 1x assay buffer. Annexin-V binding was measured under continuous magnetic stirring at 20°C in a 3 ml quartz cuvette in a Shimadzu fluorescence thermostatic spectrometer with the excitation and emission wavelengths set at 488 nm and 530 nm respectively.

Measurement of ROS production with DCF-DA

ROS production was measured using DCF-DA. In brief, HEp-2 cells were transferred to tubes and incubated with complete medium containing cariporide or PD98059 at 37°C. An equal number of HEp-2 cells was incubated without inhibitors and served as the control population. 5 µg/ml DCF-DA was added to each tube followed by incubation in the dark for 5 min at 37°C. After two washes were performed, fluorescence was measured under continuous magnetic stirring at 20°C in a 3 ml quartz cuvette using a Shimadzu fluorescence thermostatic spectrophotometer with the excitation and emission wavelengths set at 495 nm and 530 nm respectively.

Statistical analysis

Values are expressed as the arithmetic means $\pm$ standard deviations (SDs). Comparison between groups was conducted through the two-tailed paired t test and the one-way analysis of variance with the Student-Newman-Keuls test. $P < 0.05$ was used as the minimum accepted significance level. For the statistical evaluation, the statistical software GraphPad InStat version 3.00 was used (GraphPad Software, San Diego, California, (USA)).

Fig. 2. Effect of PD98059 (500nM, 5 μ M, 50 μ M) on HEp-2 cell intracellular pH. The arithmetic means of at least six experiments are shown. Error bars indicate standard deviations (SDs). The level of significance of the differences between the samples was calculated by ANOVA and Student-Newman-Keuls post-test. $P<0.05$ for the time points after and including 2h compared to the control.

Fig. 3. Effect of cariporide (20nM) and PD98059 (50 μ M) on HEp-2 cell intracellular pH in the absence of iodoacetic Na, methazolamide and DIDS. The arithmetic means of at least six experiments are shown. Error bars indicate standard deviations (SDs). The level of significance of the differences between the samples was calculated by ANOVA and Student-Newman-Keuls post-test. $P<0.05$ for the time points after and including 2h compared to the control.

Fig. 4. Na⁺ uptake by HEp-2 cells after incubation with either 20nM cariporide or 50 μ M PD98059 for 30min. The arithmetic means of at least six experiments are shown. Error bars indicate standard deviations (SDs). The level of significance of the differences between the samples was calculated by ANOVA and Student-Newman-Keuls post-test. $P<0.05$ for the samples incubated with the inhibitors compared to the control.

Results

Intracellular pH (pHi)

The intracellular pH value of the control HEp-2 population was found to be 7.33 ± 0.01 . Incubation with cariporide at three different concentrations (2nM, 20nM and 200nM) caused a significant decrease in pHi (Fig.

1). After 24h the pHi value dropped in relation to the control from between 0.23 units (with 2nM cariporide) to 0.35 units (with 20nM cariporide). Incubation with PD98059 at three different concentrations (500nM, 5 μ M, 50 μ M) had a similar effect on pHi (Fig.2). After 24h the pHi decrease was between 0.24 units (with 500nM PD98059) and 0.48 units (with 50 μ M PD98059). Any

Fig. 5. (a) ERK activity in HEp-2 cells after incubation with either 20nM cariporide or 50μM PD98059 for 30min. The arithmetic means of at least six experiments are shown. Error bars indicate standard deviations (SDs). The level of significance of the differences between the samples was calculated by ANOVA with a Student-Newman-Keuls post-test. $P < 0.05$ for the samples incubated with the inhibitors compared to the control. (b) Immunoblotting was performed as described in materials and methods. The samples incubated with cariporide and PD98059 had been incubated in FCS-containing serum.

Fig. 6. Effect of cariporide (2nM, 20nM, 200nM) on [³H] thymidine incorporation levels in HEp-2 cells. The arithmetic means of at least six experiments are shown. Error bars indicate standard deviations (SDs). The level of significance of the differences between the samples was calculated by ANOVA with a Student-Newman-Keuls post-test. $P < 0.05$ for the time points after and including 2h compared to the control.

change in pHi could be attributed only to NHE1 function as interference from any other pHi-regulatory system was appropriately inhibited (as described in Material and Methods, section 2.3). Samples were also incubated with cariporide or PD98059 in the absence of the inhibitors of the other pHi-regulatory systems leading to similar results (Fig.3).

Na⁺ uptake

Both 20nM cariporide and 50μM PD98059 had a significant effect on HEp-2 cell Na⁺ uptake after 30min of incubation, leading to decreases by 47% and 32% respectively, compared to the control Hep-2 population (Fig. 4).

ERK activity assay and immunoblot

HEp-2 cells of the control population, exhibited high levels of phosphorylation on thr185 and tyr187 and therefore activation of ERK. 50μM PD98059, as expected, practically abolished ERK activity (Fig.5). Interestingly, incubation for 30min with 20nM cariporide attenuated ERK activity, indicating NHE1 involvement in the signaling pathway. In particular, PD98059 and cariporide caused an 85% and 40.7% decrease in ERK activity respectively, compared to the control sample (Fig.5). Immunoblotting showed that ERK1 was phosphorylated at Thr202 and Tyr204 and was thus activated in HEp-2 cells incubated with FCS. Both cariporide (20nM) and PD98059 (50μM) abolished this effect (Fig.5).

Fig. 7. Effect of PD98059 (500nM, 5μM, 50μM) on [³H] thymidine incorporation levels in HEp-2 cells. The arithmetic means of at least six experiments are shown. Error bars indicate standard deviations (SDs). The level of significance of the differences between the samples was calculated by ANOVA with a Student-Newman-Keuls post-test. $P < 0.05$ for the time points after and including 2h compared to the control.

Fig. 8. Effect of cariporide (2nM, 20nM, 200nM) on ROS production levels in HEp-2 cells, as measured with DCF-DA. The arithmetic means of at least six experiments are shown. Error bars indicate standard deviations (SDs). The level of significance of the differences between the samples was calculated by ANOVA with a Student-Newman-Keuls post-test. $P < 0.05$ compared to the control for all time points except the two lower concentration samples at 0.5h and 24h.

Fig. 9. Effect of PD98059 (500nM, 5μM, 50μM) on ROS production levels in HEp-2 cells, as measured with DCF-DA. The arithmetic means of at least six experiments are shown. Error bars indicate standard deviations (SDs). The level of significance of the differences between the samples was calculated by ANOVA with a Student-Newman-Keuls post-test. $P < 0.05$ compared to the control for all time points.

[³H] thymidine incorporation

Untreated HEp-2 cells exhibited high levels of [³H] thymidine incorporation. Incubation with three different concentrations of cariporide (2nM, 20nM and 200nM) led to a decrease in [³H] thymidine incorporation by HEp-2 cells (Fig.6). This effect was most evident at 24h and particularly when the two higher cariporide concentrations were used. Incubation with three different concentrations of PD98059 (500nM, 5μM and 50μM) also resulted in

decreased [³H] thymidine incorporation by HEp-2 cells (Fig.7). In this case the effect was most evident after 5h of incubation for all concentrations used.

ROS production

Incubation with cariporide at three different concentrations (2nM, 20nM and 200nM) resulted in an increase in ROS production, which reached a peak after 2h (Fig.8). After this point, ROS production decreased,

Fig. 10. Effect of cariporide (2nM, 20nM, 200nM) on caspase-3 activation levels in HEp-2 cells. Error bars indicate standard deviations (SDs). The level of significance of the differences between the samples was calculated by ANOVA with a Student-Newman-Keuls post-test. $P < 0.05$ compared to the control for all time points except 0.5h and 24h.

Fig. 11. Effect of PD98059 (500nM, 5μM, 50μM) on caspase-3 activation levels in HEp-2 cells. Error bars indicate standard deviations (SDs). The level of significance of the differences between the samples was calculated by ANOVA with a Student-Newman-Keuls post-test. $P < 0.05$ compared to the control for all time points except 0.5h and 24h.

remaining however, above the control population levels for the next two measured time points of 5h and 7.5h. A similar, yet stronger effect on ROS production was observed after incubation with three different concentrations of PD98059 (500nM, 5μM and 50μM). ROS production was at particularly high levels for the time period between 2h and 5h (Fig.9) and remained considerably high even after 24h of incubation in relation to the control.

Caspase-3 activation

Caspase-3 activity in the control HEp-2 population was at very low levels. Incubation with cariporide at three different concentrations (2nM, 20nM and 200nM) resulted in increased caspase-3 activation, which reached a peak after 5h (Fig.10). After this point, caspase-3 activity decreased steeply at first, tending to reach the control population levels after 24h. A similar effect was observed after incubation with three different concentrations of PD98059 (500nM, 5μM and 50μM). Again caspase-3

activation peaked after 5h and then began to decrease tending to reach the control population levels after 24h (Fig.11). Cariporide had a stronger effect on initiating caspase-3 activation compared to PD98059 at the concentrations used.

DNA fragmentation

Incubation of HEp-2 cells with both 20nM cariporide and 50μM PD98059 resulted in increased levels of DNA fragmentation, compared to the control HEp-2 population. Maximum effect was observed after 7.5h of incubation (Fig.12). In particular, at this time point cariporide and PD98059 caused increases of DNA fragmentation levels by 22.1% and 17.5% respectively, compared to the control HEp-2 population.

Annexin-V binding

Incubation of HEp-2 cells with both 20nM cariporide and 50μM PD98059 resulted in increased levels of annexin-V binding, compared to the control HEp-2 population (Fig.

Fig. 12. Effect of cariporide (20nM) and PD98059 (50μM) on DNA fragmentation levels of HEp-2 cells. The arithmetic means of at least six experiments are shown. Error bars indicate standard deviations (SDs). The level of significance of the differences between the samples was calculated by ANOVA with a Student-Newman-Keuls post-test. $P < 0.05$ compared to the control for all time points except 0.5h.

Fig. 13. Effect of cariporide (20nM) and PD98059 (50μM) on annexin-V binding to HEp-2 cells. The arithmetic means of at least six experiments are shown. Error bars indicate standard deviations (SDs). The level of significance of the differences between the samples was calculated by ANOVA with a Student-Newman-Keuls post-test. $P < 0.05$ compared to the control for time points 7.5h and 24h.

13). The effect of both agents peaked after 24h of incubation. In particular, at this time point cariporide and PD98059 caused increases of annexin-V binding levels by 92.9% and 76.3% respectively, compared to the control HEp-2 population.

Discussion

NHE1, a major pH_i-regulating element is known to be activated in cancer cells leading to higher than normal pH_i levels [23]. Our data indicate that the intracellular pH of HEp-2 cells is indeed elevated. Inhibition of NHE1 for up to 24h in HEp-2 cells resulted in a significant decrease in pH_i. This decrease was observed irrespective of the inhibition or not of other pH_i-regulating systems. This data substantiates the fact that the elevated pH_i value in HEp-2 cells is a result of increased NHE1 activity. This is further supported by the results concerning Na⁺

uptake, an additional means of estimating NHE1 activity. Interestingly, inhibition of ERK under the same conditions produced an even greater decrease in pH_i compared to NHE1 inhibition, while at the same time it also attenuated Na⁺ uptake.

It has also been reported that inhibition of NHE1 in cancer cells inhibits cell proliferation and thus pharmaceutical substances such as cariporide, a potent and highly specific inhibitor of NHE1, have been proposed for use in the treatment of cancer through chemotherapy [23, 29]. In parallel to this, our data indicates that inhibition of NHE1 also leads to a significant decrease in [³H] thymidine incorporation, a common way of measuring cell proliferation. ERK activation has been associated with cell survival and proliferation [17, 30]. Our data show that ERK inhibition also attenuated [³H] thymidine incorporation.

It has been reported that inhibition of ERK leads to inhibition of NHE1 [16, 31]. Our data indicate that the

Scheme. Time course of events after inhibition of NHE1 or ERK with cariporide or PD98059 respectively.

opposite is also true in that inhibition of NHE1 attenuates ERK activity and therefore indicate to the existence of a positive feedback loop between NHE1 and ERK. The existence of this feedback loop would appear to be contradictory to the data of a previous study in which intracellular acidification induced NHE1 stimulation through an ERK-mediated pathway [17]. However, in our case either NHE1 is inhibited and therefore ERK can not mediate its stimulation or ERK is inhibited and therefore can not act upon intracellular acidification. Moreover, our data are supported by one of our previous studies in which inhibition of NHE1 with EIPA, a less specific NHE1 inhibitor compared to cariporide, resulted in ERK inhibition in non-proliferating cells as well [32]. Communication between NHE1 and ERK is probably mediated through other proteins, possibly the cytoskeleton, since it is known that NHE1-cytoskeleton interaction exists [6, 24], or even through ERK-mediated phosphorylation of NHE1 through RSK (Ribosomal S6 Kinase) as has been suggested recently [33]. To date, the exact positions of NHE1 and ERK in the signaling pathways they share are not fully understood [34].

Cytoplasmic acidification and impediment of cell proliferation are factors which can lead to apoptosis [20, 23]. Increased ROS levels have also been associated with apoptosis induction [35]. Our data indicates that following NHE1 or ERK inhibition, ROS levels increase transiently with the peak being after 2-5h. This is in contrast to previous studies where it was reported that NHE1 inhibition exerted a protective effect against ROS production [36,

37]. The possibility of a differential response depending on the cancer phenotype and cell type can not be excluded. Accordingly, we investigated the time course of the appearance of particular markers of apoptosis after NHE1 or ERK inhibition.

Both NHE1 and ERK inhibition produced caspase-3 activation peaks after approximately 5h. It is noteworthy from this time course study that the ROS production peak coincides with the caspase-3 activation peak. Additionally, NHE1 inhibition was more potent in causing caspase-3 activation compared to ERK inhibition. NHE1 is known to be a caspase-3 substrate [38]. Additionally, it has been shown that ERK can inhibit caspase-3 activation [39]. On the other hand, it has been demonstrated that caspase-3 has the ability to cleave and inactivate Raf-1, an upstream activator of ERK, resulting indirectly in ERK inhibition [40]. In summing up all the above, a plausible scenario is that inhibition of NHE1 leads to inhibition of ERK, which as a result can not halt the activation of caspase-3. After its activation, caspase-3 through its proteolytic action further inhibits NHE1 directly by cleaving it and ERK indirectly by cleaving Raf-1. Consequently, this result provides additional support to the proposed existence of a feedback loop between NHE1 and ERK.

Furthermore, DNA fragmentation and annexin-V binding, two markers of late stage apoptosis, also exhibited high levels after NHE1 or ERK inhibition. Again NHE1 inhibition was found to be more potent in inducing apoptosis compared to ERK inhibition. One can not deny the

possibility that apoptosis induced through ERK inhibition might utilize NHE1 inhibition as well. However, as our results point out, direct NHE1 inhibition was more potent in inducing apoptosis, therefore ERK inhibition must also employ alternative pathways in order for apoptosis induction.

The data of the present study report for the first time a time course of events such as ROS production, [³H] thymidine incorporation, caspase-3 activation, DNA fragmentation and annexin-V binding in relation to NHE1 and ERK inhibition in HEp-2 cells. We found that after just 30min of NHE1 or ERK inhibition, intracellular pH (pHi) levels exhibited a significant decrease. After 2h reactive oxygen species production reached a maximum and remained at high levels for at least three more hours, while pHi continued to decrease. Caspase-3 activation levels peaked after 5h. At the same time, when ERK was inhibited, DNA synthesis levels reached a minimum. At 7.5h pHi began to enter the acidic region and DNA fragmentation levels were at a maximum. Finally, after 24h, annexin-V binding levels peaked. Despite small differences concerning the timing of ROS production and minimum DNA synthesis levels, both NHE1 and ERK inhibition produced a similar time course of events (scheme) with NHE1 inhibition being more potent in

inducing apoptosis compared to ERK inhibition.

Taken together these results suggest that inhibition of NHE1 or ERK for long time periods has the capacity to induce apoptosis. In particular the inhibition of NHE1 was more potent in inducing apoptosis compared to ERK inhibition. In conclusion, the data of the present study support the existence of an apoptosis inducing signaling pathway where NHE1 and ERK play an important role. Additionally, there are indications that a positive feedback loop between the two proteins might exist. Inhibition of either one terminates this feedback loop and has the capacity to initiate apoptosis. Future research is needed in order to fully elucidate the mechanism of NHE1-ERK interaction, as well as how their inhibition induces apoptosis.

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