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Culture density and the activity, abundance and phosphorylation of the Na^+/H^+ exchanger isoform 1 in human fibroblasts

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Summary The Na^+/H^+ exchanger isoform 1 (NHE-1) is a ubiquitous membrane glycoprotein present on most eukaryotic cells. Its activity, abundance and phosphorylation are regulated by a variety of growth factors and agonists. Although cell contact and inhibition of proliferation may reduce its activity, little is known of the influence of cell culture density on these measurements. The effect of culture density on the intracellular pH (pH_i) and activity of NHE-1 of human MRC5 fibroblasts was thus investigated using fluorometry with BCECF, NHE-1 abundance with western blotting and NHE-1 phosphorylation using specific polyclonal antibodies. Proliferating cells in low density cultures had lower pH_i and NHE-1 activity (per litre of cell water) than contact inhibited confluent cultures. Such cells in low density cultures were larger than those in very confluent cultures. NHE-1 activity per cell and NHE-1 protein abundance also showed an increasing trend with culture density. However, the turnover number of NHE-1 remained at around 3000 s^{-1} in low density and sub-confluent cultures, only decreasing in very confluent cultures. Moreover, NHE-1 phosphorylation declined with increased culture density. Cell culture density has profound effects on NHE-1 activity, abundance and turnover number, with associated changes in NHE-1 phosphorylation.

The Na^+/H^+ exchanger isoform 1 (NHE-1) is a membrane transporter glycoprotein present on all eukaryotic cells, and is important for the regulation of intracellular pH and volume (1,2) in addition to a permissive role in cell proliferation. It belongs to a growing family of Na^+/H^+ exchangers, some members of which (e.g. NHE-2 and 3) are predominantly associated with epithelia and may be concerned with trans-epithelial Na^+ transport (3-5). The activity of NHE-1 is modulated by a variety of growth factors and agonists (1,6-8). Such hyperplastic and hypertrophic stimuli may have differential effects on NHE-1 mRNA levels (6). Moreover, the expression of NHE-1 also changes during cellular differentiation (9). In addition to changes in NHE-1 expression, agonists may affect protein function by post-translational processes such as phosphorylation (7,8,10).

Another possible factor that could influence NHE-1 activity is cell shape and cell to cell contact. Spreading cells have a higher intracellular pH (pH_i) than rounded cells, although no direct measurement of NHE-1 activity was made (11). Cells actively growing at the

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periphery of a colony of renal epithelial cells have a lower pH_i and as well as lower expression of Na^+/H^+ exchanger (NHE) activity (12). Cell culture density may thus affect NHE activity, although effects on NHE-1 protein content, exchanger turnover number and phosphorylation have not been defined. Due to the increasing use of cultured cells for investigation of NHE function in hypertension (13) and diabetes (14), differences between patient groups have been reported but studies have often not controlled for differences in cell culture density.

We have therefore studied the effects of growth of human MRC5 fibroblasts at different densities on the pH_i and NHE-1 activity, together with measurements of NHE-1 abundance in these cells. Previously, it has been established that mRNA transcripts of NHE-2 and NHE-3 are not abundantly present in fibroblasts (4,5), so that the predominant isoform in these cells is NHE-1. This is also confirmed by the sensitivity of NHE activity in fibroblasts to inhibition by ethyl-isopropyl amiloride (15). Turnover numbers for NHE-1 could be estimated since the majority of NHE-1 is plasma membrane associated, with little intracellular compartmentalization (15,16). Since phosphorylation may play a role in the activation of NHE-1 (7,8,10), the effect of culture at different densities on NHE-1 phosphorylation was also determined. Our findings suggest that cells growing at low culture densities had reduced pH_i , reduced NHE activity per cell together with reduced NHE-1 protein content per cell. Turnover number remained constant until high densities were achieved. These changes were associated with elevated NHE-1 phosphorylation in low density cultures.

Materials and Methods

Reagents 2,7' Bis-(carboxyethyl)-5,6-carboxyfluorescein acetoxymethyl ester (BCECF-AM), bovine serum albumin (BSA), glutamine, nigericin, monensin and polyoxyethylene-8-lauryl ether were purchased from Sigma Chemicals Ltd., Poole, Dorset, U.K. Media and reagents for cell culture were from Gibco BRL, Life Technologies Ltd., Uxbridge, Middlesex, U.K. The DMEM growth medium contained 10 % FCS, 2 mM L- glutamine and penicillin/streptomycin (10^5 U of each / l). Protein A Sepharose CL4B, glutathione Sepharose 4B and the pGEX-2T plasmid were from Pharmacia LKB Biotechnology, Uppsala, Sweden. ^{3}H-3-O-methyl-D-glucose, Hybond C-extra nitrocellulose and enhanced chemiluminescence (ECL) western blotting reagents were supplied by Amersham International, Little Chalfont, Buckinghamshire, U.K. ^{32}P-orthophosphate was obtained from ICN Biomedicals Ltd., Thame, Oxfordshire, U.K.

Cell culture MRC5 fibroblasts were obtained from the European Collection of Animal Cell Cultures, Porton Down, Salisbury, U.K. and were cultured in DMEM growth medium. All data on MRC5 fibroblasts are derived from cells after 20-30 passages. In order to obtain cells at different culture densities, we seeded the fibroblasts into tissue culture flasks so that the equivalent area covered by the cells was (a) 90 %, (b) 25 % and (c) 15 % on the day of seeding. After culture in DMEM growth medium for 5 days, the flasks were judged to be (a) very confluent (100 % culture area covered), (b) subconfluent (90-95% area covered) or (c) 50 % confluent respectively. These cells were then used for measurements of NHE-1 abundance, cell volumes and NHE-1 phosphorylation as described below without a prior period of serum withdrawal.

Measurement of intracellular pH and Na^+/H^+ exchanger activity Fibroblasts were seeded onto 9x22 mm glass coverslips, using the protocol described above, to obtain cells adherent to cover slips with differing culture densities. The cover slips were incubated with

BCECF-AM (5 μM in TC199) at 37°C for ½ hour (14,15). After complete de-esterification of the dye, measurements of intracellular pH (pH_i), intrinsic buffering capacity and H^+ efflux were performed at 37°C, as described in detail previously (14-16).

Estimation of NHE-1 abundance in fibroblasts NHE-1 specific polyclonal antibodies G252 and G253 are protein-A Sepharose partially purified immunoglobulin fractions from antisera raised against a β- galactosidase -NHE-1 C-terminal fusion protein containing the final 157 amino acids of NHE-1 (7,15,16). A glutathione S-transferase NHE-1 C-terminal fusion protein (GST fusion protein) was also constructed so that the same amino acids of NHE-1 from the original β-galactosidase fusion protein were present (15). In order to estimate NHE-1 abundance per cell, known numbers of cells were extracted and detected with NHE-1 specific G252 antibody as described (15,16). Bands corresponding to NHE-1 (approximately 97 kD) were quantitated with a BioRad densitometer (BioRad Laboratories Ltd., Hemel Hempstead, Hertfordshire, U.K.). Titration with different amounts of the GST fusion protein enabled us to estimate the NHE-1 abundance in cell extracts (15) using a value of 6.022×10^{23} as Avogadro's constant. Turnover numbers were computed from these values of NHE mediated H^+ efflux per cell and NHE-1 protein content per cell. Cell volume was measured using the ^{3}H-3-O-methyl-D-glucose technique (17).

Immunoprecipitation of ^{32}P labelled NHE-1 from fibroblasts The fibroblasts cultured at different densities were washed three times in phosphate free HEPES buffered saline (HBSS) composed of (in mM) NaCl 130, KCl 5, $CaCl_2$ 1.8, $MgSO_4$ 1, glucose 5, HEPES 20, glutamine 2, BSA 1 g/l, pH 7.4. 50 μCi/ml of carrier free ^{32}P- orthophosphate was then added to the flasks followed by incubation for 3 h at 37°C. Labelled cells were washed briefly with cold HBSS and snap frozen with liquid nitrogen. One ml of cold (4°C) extraction buffer composed of 10 g/l polyoxyethylene-8-lauryl ether , (in mM) Tris 30, NaCl 130, EDTA 5, phenylmethylsulphonyl fluoride 1, o-phenanthroline 1, iodoacetamide 1, Na fluoride 100 , Na orthovanadate 5, ATP 10, Na pyrophosphate 10, (in mg/l) pepstatin A 1, leupeptin 2 was then added to the frozen monolayer. The cells were then scraped off, the suspension sonicated and cell debris was then removed by centrifugation at 14,000 g. The supernatant was then preabsorbed with protein A -Sepharose CL4B beads. The NHE-1 specific polyclonal antibody G253 was added to the supernatant at a final concentration of 100 μg/ml and the samples rotated end on end for 2 h at 4°C. Protein A-Sepharose-CL4B beads that had been pretreated with unlabelled fibroblast extracts to reduce non-specific binding were then added for a further 1 h to recover the antibody. After extensive washes, the beads were boiled in Laemmli sample buffer for 3 min and phosphoproteins were resolved on 7.5 % SDS-PAGE gels. Detection was by autoradiography on pre-flashed X-ray films at -70°C. Densities of the ^{32}P labelled NHE-1 were determined using a BioRad densitometer. Concurrent measurement of NHE-1 abundance by western blotting of extracts from similar flasks enabled the calculation of a phosphorylation index per unit NHE-1 protein.

Measurement of ^{32}P-ATP specific activity Cells were labelled as for immuno-precipitation using 0.5 μCi/ml of carrier free ^{32}P- orthophosphate. Labelled cells were washed briefly with cold HBSS and snap frozen with liquid nitrogen. The cells were scraped off into one ml of cold 10mM Tris pH 7.4 containing 5mM EDTA and debris removed by centrifugation at 13,000 g for 10 minutes. ATP specific activity was determined by high pressure liquid chromatography using a 10SAX Partisil anion column and eluting with a linear gradient of 0 - 67% 1.4 M ammonium dihydrogen orthophosphate. Total ATP levels were determined by absorption at 254 nm with reference to known amounts of ATP and ^{32}P incorporation by Cerenkov counting the fractions obtained on chromatography.

Statistics Results are expressed as means ± SEM, and comparisons were by ANOVA and Student's t test, performed on an Oxstat statistics package (Microsoft Corporation, Reading, U.K.). Two tailed P values under 0.05 were considered significant.

Results and Discussion

NHE mediated H^+ efflux and NHE-1 protein content at different culture densities

Table 1 illustrates the differences in DNA content achieved by the above cell seeding protocol, so that the confluent flasks contained approximately 1.8 to 3 times as many cells as the subconfluent and 50 % confluent flasks respectively. Cell volumes in 50 % confluent and subconfluent cultures were significantly larger than very confluent cultures.

Measurements of pH_i of cells seeded at different densities produced the lowest values in 50 % confluent cultures, with more alkaline pH_i as density increased (Table 1). The lower pH_i values in low density cultures may be related to higher H^+ production rates in actively proliferating cells. Buffering measurements and passive H^+ efflux rates were very similar at all culture densities (Table 1). NHE activity (calculated as the product of buffering capacity and Na^+ dependent dpH_i /dt and expressed per volume of cell water) was lowest in the 50 %

Table 1 Resting intracellular pH, buffering capacity and H^+ efflux mechanisms at pH_i 6.0 and NHE-1 abundance in MRC5 fibroblasts seeded at different cell densities (n = 12 for pH, buffering, flux and NHE-1 turnover measurements and n = 6 for NHE-1 abundance measurements). Means ± SEM are reported.

	50% confluence	Subconfluent	Confluent
DNA content per 75 cm^2 flask (μg)	6.75 ± 0.48[b]	11.27 ± 1.72	20.07 ± 2.67[a]
Cell volume (fL)	4265 ± 211	3813 ± 262	2324 ± 186[b]
pH_i	6.92 ± 0.03[a]	7.05 ± 0.02	7.31 ± 0.03[c]
Buffering capacity at pH_i 6.0 (mM/pH)	80.2 ± 5.8	80.9 ± 2.9	79.5 ± 3.0
Na^+/H^+ exchanger H^+ efflux (mM/min)	21.3 ± 1.1[c]	38.1 ± 2.3	80.0 ± 3.0[c]
Na^+/H^+ exchanger H^+ efflux per cell (fmol/min)	90.7 ± 4.7[c]	145.4 ± 8.9	185.9 ± 7.0[b]
NHE-1 abundance (sites/cell)	299326 ± 29725[a]	447570 ± 28107	749042 ± 40316[b]
Turnover rate of NHE-1 (cycles/s)	3041 ± 157	3259 ± 200	2491 ± 93[b]

a P<0.01 compared to subconfluent values.
b P<0.005 compared to subconfluent values.
c P<0.001 compared to subconfluent values.

confluent cultures, with higher values in both the subconfluent and confluent cultures (Table 1). NHE-1 mediated H^+ efflux expressed on a per cell basis showed the same rising trend as culture density increased.

Figure 1 shows a typical western blot of extracts from cells cultured at the different densities stated above. NHE-1 specific polyclonal antibody G252 detected a band of approximately 97 kD, consistent with the molecular weight of glycosylated human NHE-1, as reported previously (7). The highest amounts of NHE-1 protein (per cell) were present in the very confluent cultures, with progressively less NHE-1 protein per cell in less confluent cultures. There were no significant molecular weight differences in NHE-1 between cultures of different densities (Fig. 1). The number of NHE-1 sites per cell were calculated using a serial dilution of GST fusion protein as described previously (15), confirming a 2.5 fold difference in NHE-1 sites per cell between the 50 % confluent and the very confluent cultures (Table 1). With the present data, it was not possible to express NHE activity per unit area of membrane, since these cells at different densities have diverse shapes making it difficult to estimate the membrane area per cell.

The turnover number for NHE-1 was computed from the above measurements of NHE activity per cell and NHE-1 protein abundance per cell. Turnover numbers were higher in the proliferating cells (50 % confluent and subconfluent cultures) falling as higher culture densities were achieved (Table 1).

NHE-1 phosphorylation and cell culture density

Phosphorylation is one mechanism whereby NHE-1 activity could be modulated by growth factors or phosphatase inhibitors (7,8,10). We therefore immunoprecipitated NHE-1 from extracts of cultures of different densities prelabelled with ^{32}P- orthophosphate. Figure 2 shows a typical experiment demonstrating that the highest NHE-1 phosphorylation occurred in those cultures of lowest density (50 % confluence) where the cells are most actively

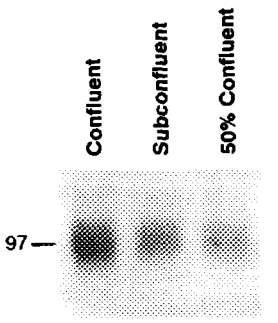

Fig. 1. A typical western blot of cell extracts from cultures that were confluent, subconfluent or 50 % confluent. Extracts from 10^5 cells were loaded onto each lane and resolved on a 7.5 % SDS PAGE gel before electroblotting onto supported nitrocellulose membranes and detection with the NHE-1 specific antibody G252. The molecular weight marker for 97 kD is shown.

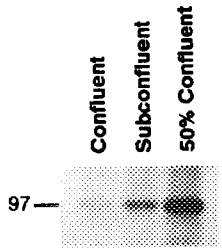

Fig. 2. Cells at different densities were labelled with 50 μCi/ml of carrier free ^{32}P-orthophosphate in phosphate free HBSS. After 3 h, the monolayers were frozen in liquid nitrogen and extracted, before immunoprecipitation with NHE-1 specific antibody G253. Phosphoproteins were resolved on 7.5 % SDS PAGE gels before autoradiography. The molecular weight marker for 97 kD is shown.

proliferating and not contact inhibited. After correction for differences in NHE-1 protein amount and ^{32}P-ATP specific activities (measured on HPLC), the relative degree of phosphorylation per NHE-1 protein site was 1.00 ± 0.02 for confluent cells, 3.23 ± 0.19 for subconfluent cells and 5.30 ± 0.19 for the 50% confluent cultures ($p<0.01$ relative to confluent cultures, $n = 3$).

These studies clearly demonstrate that NHE-1 activity rises (whether expressed as per volume of cell water or per cell) as culture density increases. Moreover, this occurs with a concurrent increased expression of NHE-1 protein. The cells at low density are likely to be actively proliferating compared to those that are contact inhibited at higher densities. In contrast to what may have been expected (that is, a higher NHE activity and expression in the cells that are proliferating most actively), the opposite trend has been documented in the present studies. However, actively proliferating cells at lower densities also had higher NHE-1 turnover numbers and higher NHE-1 phosphorylation compared to the confluent cultures. These two parameters may be more closely linked to cell proliferation than NHE activity or NHE-1 protein content *per se*. These findings may have implications for basic research on NHE-1 activity and expression, as well as on the role of NHE-1 in the pathophysiology of hypertension (13) and diabetic nephropathy (14). Future studies on cellular NHE-1 activity or its regulation by phosphorylation in basic or clinical studies will need to control for the density of such cultured adherent cells as differences in these parameters could be dependent on the degree of confluence of these cultures.

Acknowledgments

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