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NHERF-1 regulation of EGF and neurotensin signalling in HT-29 epithelial cells

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ABSTRACT

Neurotensin receptors (NT-R) and the epidermal growth factor receptors (EGF-R) are commonly overexpressed in many epithelial origin tumours. In addition to their role as mitogenic mediators through specific cell signalling, recent studies indicate that the activity/expression of scaffold proteins responsible for the assembly and coordination of the signalling complexes may also have central roles in epithelial transformation. In particular, the "epithelial" PSD-95/Dlg/Zo-1 (PDZ) scaffold/adapter protein, Na^+/H^+ exchanger regulatory factor isoform one (NHERF-1), has been identified as a potential regulator of cellular transformation. NHERF-1 is a known regulator of EGF-R function and plays numerous roles in G-protein-coupled receptor signalling. Because of the synergistic signalling between these two potent mitogens, we investigated a potential role for NHERF-1 in the molecular mechanism linking the aberrant proliferative phenotype initiated by some G-Protein-coupled receptor activators in the colon adenocarcinoma HT-29 cell line. Knockdown (80%) of endogenous NHERF-1 leads to significant reduction in proliferation rate; an effect that could not be recovered by exogenous application of either NT or EGF. Inhibition of the EGF-R with AG1487 also inhibited proliferation and this effect could not be recovered with NT. Knockdown of NHERF-1 significantly altered the expression of the EGF-R, and almost completely abolished the NT-mediated increases in intracellular free Ca^{2+}. Knockdown of NHERF-1 also attenuated UTP-mediated purinergic Ca^{2+} signalling. Taken together, these data suggest that NHERF-1 plays a more central role in cell proliferation by modulating Gq-mediated signalling pathways.

1. Introduction

Transformation of colonic epithelial cells is associated with a loss of polarity, unregulated proliferation and invasion of underlying tissues. There have been several potent pro-proliferative mediators identified in gastric and colonic epithelia; these include epithelial growth factor (EGF) and neurotensin (NT) [1–3]. Changes in the signalling pathways mediated by these mitogens are frequently associated with the transformation to a malignant phenotype. In addition to mitogenic mediators, it has been recently appreciated that altered activity/expression of scaffold proteins responsible for the assembly and coordination of receptor signalling complexes may also have central roles in epithelial transformation and cancer progression. In particular, the "epithelial" PSD-95/Dlg/Zo-1 (PDZ) scaffold/adapter protein, Na^+/H^+ exchanger regulatory factor isoform one (NHERF-1), has been identified as a potential regulator of cellular transformation [4].

Altered levels of NHERF-1 correlate to tumorigenicity in the breast, schwannomas and hepatocytes [5–7]. Interestingly, there are reports of both overexpression and loss of NHERF-1 depending on the cancer cell type. In some instances this may be related to the subcellular localization of the protein [8]. Studies in breast suggest that NHERF-1 acts as a tumour suppressor when at the membrane and as a tumour promoter when over-expressed in the cytoplasm [4,5]. NHERF-1, through interactions with the cytoskeleton, by maintaining distinct temporal and spatial distribution of signalling molecules helps maintain correct cellular polarity [9], the loss of which is a feature of early tumour formation [10]. Increased cytoplasmic NHERF-1 expression in colonic epithelia can intensify the transformed phenotype and increase cell proliferation [10].

Structurally, NHERF-1 contains two PDZ modules that bind target motifs on interacting proteins and a C-terminal merlin-ezrin-radixin-moesin (MERM) binding domain that can anchor protein complexes to the cytoskeleton [11]. Known membrane protein targets of NHERF-1 include a number of G-protein coupled receptors (GPCRs) such as the κ-opioid receptor, the β-adrenergic receptor as well as growth factor receptors such as the platelet-derived growth factor receptor and EGF-R [12–15]. NHERF-1 has also been shown to indirectly regulate other GPCRs through its association with the numerous signalling molecules involved in the cascade such as PLCβ and Gq_α [16,17].

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This raises the question as to how perturbations in the level of NHERF-1 could contribute to tumour progression. One explanation lies in NHERF-1 binding partners, which include oncogenic signalling molecules such as β-catenin and phosphatase and tensin homolog (PTEN) [18,19]. In normal colonic cells, NHERF-1 is primarily associated with the plasma membrane [10]. An increased level of NHERF-1 in the cytoplasm may establish ''abnormal'' interactions not present in normal cells. For example, ectopic cytoplasmic expression of NHERF-1 in NHERF-1 depleted Caco-2 colonic epithelial cells increased cellular proliferation. It was suggested that the mechanism for this involved NHERF-1 associating with β-catenin outside the ''normal'' plasma membrane compartment thereby conferring a growth advantage [10].

A major NHERF-1 interacting receptor is EGF-R, a receptor required for normal cell growth [15]. However, overexpression of EGF-R is widely reported as a common feature of many human carcinomas. Anti-EGF-R therapy is a suggested approach to treatment of several epithelial tumours including those of the colon [20]. The EGF-R can be activated by its own ligand EGF or transactivated by several GPCRs such as the endothelin-1, lysophosphatic acid and neurotensin receptors (NT-R) [21]. NT is a 13 amino acid peptide highly expressed in the gastrointestinal tract and plays several roles including regulation of motility, secretion, inflammation and importantly, the growth/regeneration of the intestinal mucosa [22]. Activation of NT-R (1-2) results in typical GPCR signals including Ca^{2+} mobilisation and stimulation of MAPK pathways. In cell culture models of epithelial cancer, disruption of NT receptor activation with the specific agonist SR 48692 significantly attenuated cell proliferation [23]. Activation of NT receptors can also transactivate EGF-R and downstream MAPK pathways. This can induce a time-dependent increase in EGF-R gene expression due to a MAPK-mediated increase in the expression of transcription factor early growth response gene-1 (Egr-1) and enhanced binding of Egr-1 to the EGF-R promoter [24]. Thus there is a clear but complex synergism between NT and EGF signalling in epithelial cells. The HT-29 colon adenocarcinoma cell line is a widely used cell model for the study of signalling pathways involved in epithelial cancers that express both the NT-R and EGF-R [25,26]. In this study we investigated if there was a central role for NHERF-1 in EGF and NT-induced cell proliferation in the HT-29.

2. Methods and materials

2.1. Cell culture, transfection, lentivirus production and infection

The human colorectal adenocarcinoma cell line HT-29 (ATCC, Virginia, USA) were maintained in standard RPMI 1640 medium (Invitrogen, Groningen, the Netherlands), 10% turbo calf serum (Invitrogen) and 1% (v/v) penicillin/streptomycin (Invitrogen) at 37 °C, 5% CO_2. For proliferation and Ca^{2+} measurements HT29 Cells were seeded in 96 well plates at 2.5×10^4 cells and grown till approximately 50% confluent before virus infection. 293FT virus packaging cells (Invitrogen) were grown and maintained as described by the manufacturer. The shRNA constructs (TRCN0000068583 [A6], TRCN0000068584 [A7], TRCN000006858 [A8] and TRCN0000068586 [A9]) for NHERF-1 knock down were obtained from the RNAi Consortium via Open Biosystems (Huntsville, AL). All virus production and cell infections were performed as described previously [27]. Transfections were performed using Lipofectamine 2000 (Invitrogen) according to manufacturer's instructions.

2.2. Confocal microscopy

HT-29 cells were grown on glass coverslips and fixed in 4% paraformaldehyde. Immunohistochemisty was performed as previously described [28]. Briefly, cells were blocked for 1 h at RT in blocking buffer (3% normal horse serum, 0.5% BSA and 0.05% Triton-X 100). NHERF-1 antibody (1:500) was applied in blocking buffer for 1 h RT followed by incubation with Alexa 555 secondary Ab (Molecular probes, 1:500) for 30 min. Cells were mounted with prolong gold containing DAPI (Invitrogen). Cells were visualized using a Nikon C1 confocal microscope using a 405 nm violet diode and 543 nm HeNe lasers (Nikon Group, Tokyo, Japan). Images were taken using a 40× Plan-Apochromat oil immersion objective. All images were captured and analysed using Nikon Elements software.

2.3. Antibodies and western blot

Generation of the specific antibodies against NHERF-1 and -2 has been previously described [29]. The monoclonal EFGR antibody was from Cell Signalling and β-actin antibody from Sigma. Antibodies were used at the following dilutions (NHERF-1-2 1:5000, EGF-R 1:500, b-actin 1:10,000). Cells were solubilised in 150 mM NaCl, 20 mM HEPES, 1 mM EDTA, 1% NP-40 (Roche diagnostics, Penzberg, Germany), complete protease inhibitor (Roche Diagnostics) and equally loaded samples resolved on 10% SDS–PAGE gel. Proteins were transferred to nitrocellulose membrane and incubated with appropriate antibody. Immunoreactive proteins were detected by enhanced chemiluminescence (Pierce Biotechnology Inc.).

2.4. Cell cytotoxicity and proliferation assays

To assess the proliferative/anti-proliferative effect in HT-29 cells an MTT cell proliferation (Sigma–Aldrich, USA) assay was used. To determine the potential cytotoxic effects of lentiviral infection and protein knockdown, a lactate dehydrogenase (LDH) assay (Sigma) was used. All assays were performed according to manufacturer's protocols. Both control and virus infected cells were serum starved for 24 h, then incubated with EGF (10 ng/mL, Sigma) or NT (100 nM, Sigma) for 24 h. The EGF inhibitor AG1487 was dissolved in DMSO and purchased from Calbiochem (Mecrk Biosciences). This compound was incubated for 24 h in the presence and absence of NT.

2.5. $[Ca^{2+}]_i$ measurement

The intracellular cytosolic free Ca^{2+} concentration was measured after the addition of NT or uridine tri-phosphate (UTP, Sigma) (100 μM) using the fluorescence imaging plate reader FLIPR-TETRA (Molecular Devices) and the Ca^{2+}-sensitive dye Fluo-4 AM (Molecular Probes, Invitrogen) as previously described [30]. Responses were measured at 470–495 nm excitation and 515–575 nm emission over a 125 s period. Relative $[Ca^{2+}]_i$ was determined by comparing the response over baseline.

2.6. Statistical analysis

Statistical significance was assessed using ANOVA with a Tukey–Neuman post test. Data were considered statistically significant when $P < 0.05$. Unless otherwise specified, results are presented as mean ± SEM.

3. Results and discussion

3.1. NHERF-1 expression and cell growth in HT-29 cells

In light of recent reports of increased levels of NHERF-1 in proliferative colon carcinomas, we first investigated the

distribution of endogenous NHERF-1 in HT-29 carcinoma cells. In cells grown in normal serum containing medium, NHERF-1 staining was abundant and distributed throughout the cytoplasm, with little staining evident in the nucleus (Fig. 1A and B). The distribution of NHERF-1 was not significantly altered when the cells were serum starved (Fig. 1C and D). In contrast to "normal" colonic epithelial cells, where NHERF-1 is localised primarily to the apical membrane [9], our data suggested that NHERF-1 was constitutively "de-localised" in HT-29 cancer cells. This finding is consistent with the hypothesis that elevated levels of NHERF-1 and its cytosolic distribution is a key feature of the colon cancer cell phenotype.

We next investigated if reducing the levels of NHERF-1 altered the rate of HT-29 cell proliferation. Cells that were infected with Lenti-A9 showed a pronounced reduction in endogenous NHERF-1 protein with no effect on the closely related protein NHERF-2 (Fig. 2A and B). NHERF-1 was reduced by 81 ± 6% of control levels ($^{***}P < 0.001$; $n = 3$; Fig. 2C). This reduction in NHERF-1 was associated with significant changes in the growth parameters of HT-29 cells. Under normal conditions, HT-29 cells do not exhibit typical contact inhibition, rather they grow in a disordered manner with a tendency to overgrow with areas of cell death and necrosis at the monolayer margins (Fig. 2D). In contrast, cells with reduced NHERF-1 grew more slowly in culture and had a more organised monolayer suggestive of contact inhibition (Fig. 2E). To determine if the changes we observed were due to reduced proliferation or cell death we performed MTT viability/proliferation and LDH cytoxicity enzymatic assays. The LDH assay revealed that there was no difference in cell death in the NHERF-1 silenced cells compared to the wild type cells leading to the conclusion that the virus infection itself did not exert cytotoxicity (Fig. 2F). There was, however, a pronounced difference in the rates of proliferation, with the MTT assay revealing that in cells silenced for NHERF-1 the rate of proliferation was approximately half that of the control cells (reduced by 57 ± 12%; $^{**}P < 0.01$; $n = 5$; Fig. 2G). This result was supported by cell counts performed in parallel experiments showing a similar 50% reduction in cell numbers (data not shown). This highlights the central role that NHERF-1 may play in the transformed phenotype in colon carcinoma. It is interesting to note that NHERF-1$^{-/-}$ mice do not have any changes in rates of tumour development compared to wild types [19].

3.2. NHERF-1 and EGF/NT-mediated proliferation in HT-29 cells

NHERF-1 is a known scaffold for EGF-R. We hypothesised that the effect of NHERF-1 on proliferation could be explained by a disruption of normal EGF mitogenic signalling. Exposure of HT-29 cells to EGF (10 ng/ml) increased the rate of proliferation by 37 ± 9% ($^{**}P < 0.01$; $n = 4$) above control. Similarly NT (100 nM) increased proliferation by 22 ± 5% ($^{*}P < 0.05$; $n = 4$). When the cells were exposed to EGF and NT together there was no additive effect on proliferation ($n = 4$; Fig. 3A), suggesting that the two mitogens were acting predominately via a common signalling pathway. Treatment of the cells with the EGF-R inhibitor AG1487 (250 nM) inhibited proliferation by 63 ± 5% ($^{**}P < 0.01$; $n = 3$; Fig. 3B). In the presence of AG1487, treatment with NT had no effect on cell proliferation (Fig. 3B). This suggested that the mitogenic effects of NT in HT-29 cells were mediated primarily via transactivation of EGF-R. Similar results have been reported in PC3 prostate cancer cell lines where NT evoked DNA synthesis was inhibited in a concentration dependent manner by a closely related EGF inhibitor compound AG1478 [31]. However, this NT evoked response could not be elicited using EGF neutralising antibodies in either PC3 [31] or NCM460 colonocytes, pointing to a transactivation process initiated by NT [20].

We next investigated the involvement of NHERF-1 in the EGF and NT-mediated proliferative responses. Control cells showed normal proliferative responses, while cells silenced for NHERF-1 showed a strong decrease in cell proliferation as described above. Neither exogenous EGF nor NT was able to induce any proliferative response in NHERF-1 knockdown cells (Fig. 3C and D). The expression of the EGF-R is self-regulating in that increased activity of the receptor leads to its down/upregulation [15]. When HT-29 cells were exposed to either EGF or NT, the total EGF-R level was significantly increased (Fig. 3E). A major role of NHERF-1 is to stabilise the EGF-R at the cell surface by slowing the rate of endocytosis, thereby increasing the size of the pool of receptors at the cell membrane [15]. This led us to hypothesise that silencing NHERF-1 would attenuate EGF signalling with a resultant down-regulation of the receptor. Cells silenced for NHERF-1 had a pronounced reduction in the levels of EGF-R to 70 ± 10% of control ($^{**}P < 0.01$; $n = 3$). When the NHERF-1 silenced cells were exposed to either EGF or NT, EGF-R protein was almost totally down

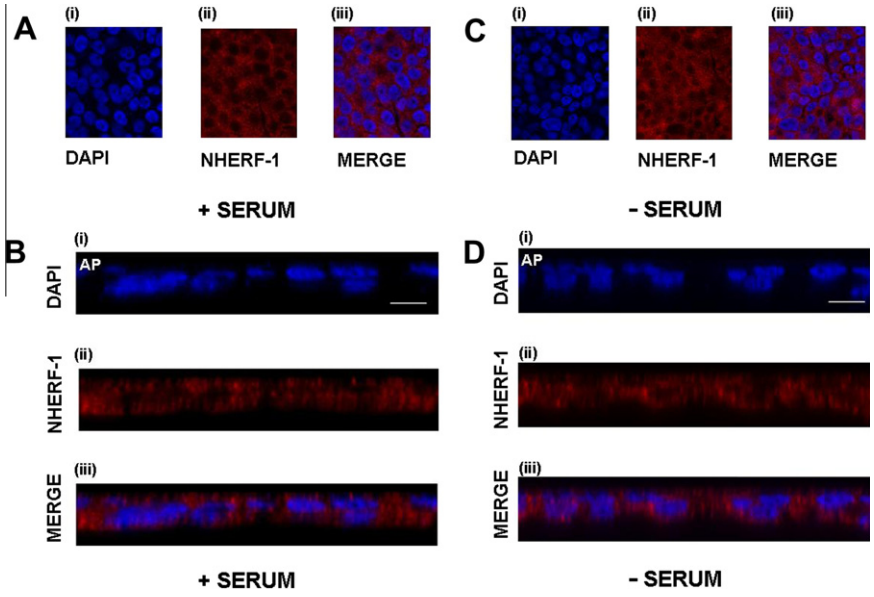

Fig. 1. Distribution of NHERF-1 in HT-29 cells. (A and B) HT-29 cells stained for NHERF-1 show abundant cytosolic labelling. (C and D) A similar pattern of staining was apparent when the cells were serum starved. Representative of three independent experiments.

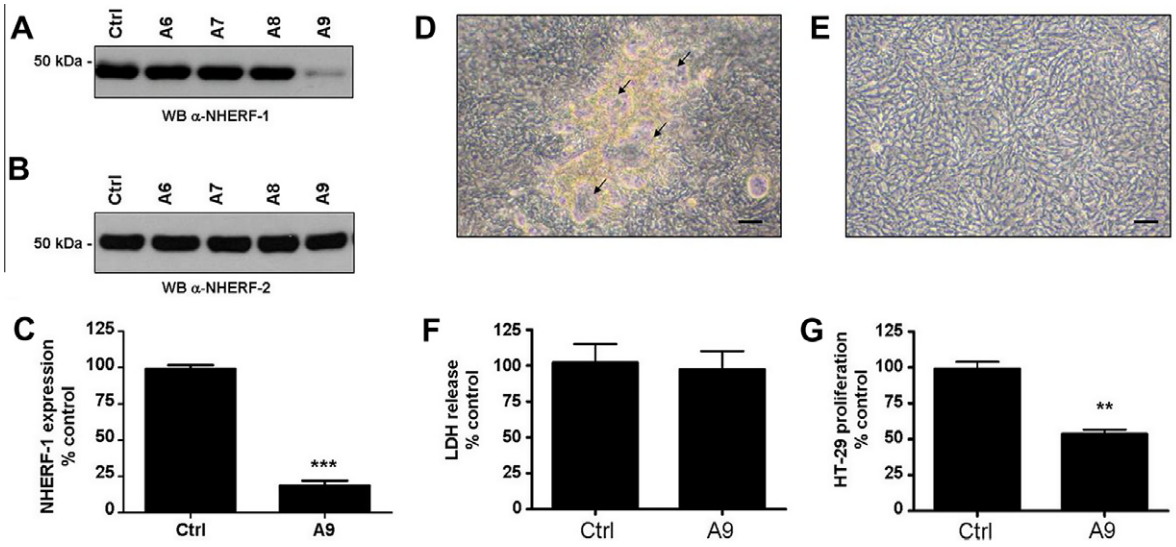

Fig. 2. Lentivirus mediated knockdown of NHERF-1 in HT-29 cells. (A) Representative western blot of HT-29 cells infected with lentiviruses against NHERF-1. (B) Representative western blot showing the closely related NHERF-2 was not affected by these viruses. (C) Virus A9 caused a highly significant knockdown (80 ± 5%; $n = 3$) of endogenous NHERF-1. (D) Phase contrast image of control infected HT-29 cells. (E) Cells infected with the A9 virus showed apparent decreased proliferation and more ordered monolayer. (F) Infection with the A9 virus significantly decreased cell proliferation (57 ± 12%) as measured by MTT assay. (G) LDH release assay showed virus infection had no cytotoxic effect on the cells. Representative of three individual experiments, expressed as mean ± SEM; $^{**}P < 0.01$; $^{***}P < 0.001$.

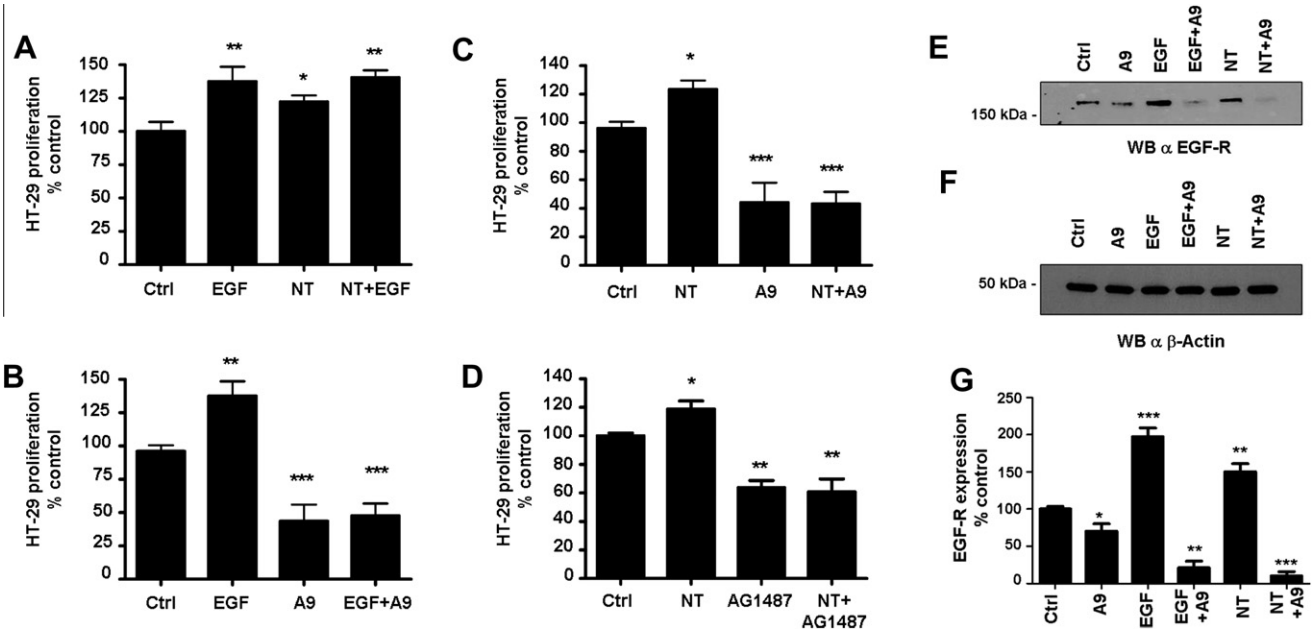

Fig. 3. Silencing of NHERF-1 abolished the EGF and NT mediated proliferative responses and attenuates EGF-R expression. (A) HT-29 cell proliferation was significantly stimulated by both EGF and NT, with no additive effect observed when the two mitogens are combined. (B) Cells inhibited with the EGF-R inhibitor AG1487 displayed a reduction in proliferation which could not be reversed by NT. (C) Cells infected with the A9 virus showed decreased proliferation and the proliferative response to NT was abolished (D) Cells infected with the A9 virus showed decreased proliferation and the proliferative response to EGF was abolished. (E) Representative western blot showing change in the expression of the EGF-R in HT-29 cells. (F) Representative western blot of β-actin loading control. (G) Densitometry analysis showing both EGF and NT both increase the expression of the EGF-R by 2- and 1.5-fold, respectively, and this is blocked when cells are silenced for NHERF-1. Representative of three individual experiments, expressed as mean ± SEM; $^{**}P < 0.01$; $^{***}P < 0.001$.

regulated (Fig. 3E and F). The mechanism for this effect remains to be elucidated but could involve decreased synthesis or enhanced degradation of EGF-R. Hence, NHERF-1 appears to play a central and complex role in maintaining the integrity and capacity of the EGF-R proliferative signalling pathways in HT-29 cells. In the absence of NHERF-1, the entire pathway appears to shutdown and the cells revert to a less proliferative phenotype.

3.3. NHERF-1 and NT Ca^{2+} signalling

The NT-R-1 on its intracellular c-terminus does not have any apparent classical PDZ binding motif. In preliminary experiments, we were unable to show any direct interaction with NHERF-1 (data not shown). NT receptors are GPCRs that signal predominantly via transient increases in $[Ca^{2+}]_i$. We therefore investigated if NHERF-1

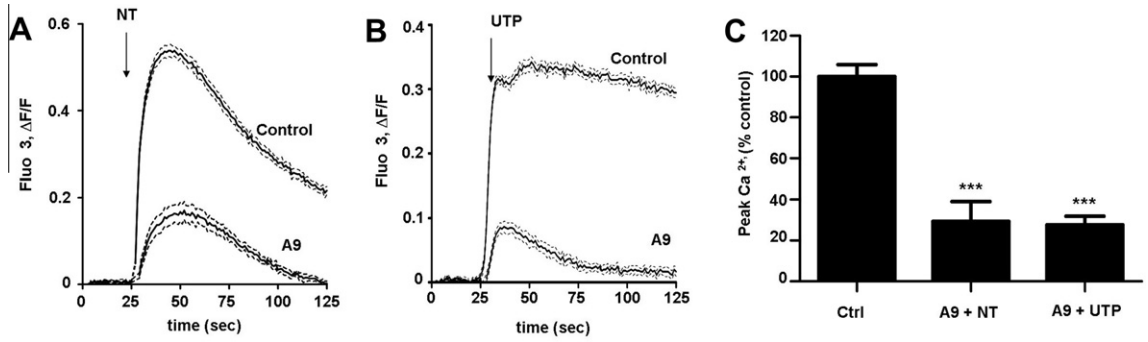

Fig. 4. Silencing of NHERF-1: effects on Gq coupled Ca^{2+} signalling in HT-29 cells. (A) Fluo-4 tracings showing the changes in $[Ca^{2+}]_i$ following NT exposure in NHERF-1 knockdown and control cells. (B) Fluo-4 tracings showing the changes in $[Ca^{2+}]_i$ following UTP exposure in NHERF-1 knockdown and control cells. (C) In NHERF-1 silenced cells, there was a significant attenuation in the increases in $[Ca^{2+}]$i mediated by either NT or UTP. Representative of three individual experiments, expressed as mean ± SEM; $^{**}P < 0.01$; $^{***}P < 0.001$.

had any regulatory role in mediating the Ca^{2+} signal generated by NT. When HT-29 cells were exposed to NT, there was the typical transient Ca^{2+} response that has been shown previously to be mediated Gq/PLCβ coupled signalling [32]. However, in cells silenced for NHERF-1, the response was significantly reduced by 70 ± 5% ($^{***}P < 0.001$; $n = 5$) (Fig. 4A and C). This suggested that NHERF-1 may also be involved at a more common step of the Gq-mediated Ca^{2+} pathway. NHERF-1 has a demonstrated role in GPCR receptor signalling and many of its binding partners are components of Gq-coupled Ca^{2+} signalling pathways such as PLCβ, protein kinases C and D and even Gq_α itself [33]. NHERF-1 interacts preferentially with activated Gq_α has been shown to influence signalling of the GPCR, thromboxane A2β (TP β) [17]. NHERF-1 does not directly bind TP β; however, the subsequent formation of a complex with the activated Gq_α causes receptor stabilization at the membrane [34]. In the case of HT-29 cells, the presence of excess NHERF-1 and buffering of activated Gq_α may perhaps have a more general effect on stabilization of GPCRs at the membrane and prolongation of agonist responses. Thus silencing NHERF-1 and removing this stabilising mechanism may attenuate any NT evoked Ca^{2+} response. In order to demonstrate that silencing NHERF-1 had a more general effect on Gq signalling, we tested the effects of silencing NHERF-1 on UTP mediated activation of Ca^{2+}. HT-29 cells express only the P_2Y_2 UTP receptors [35] and we found that the UTP mediated Ca^{2+} response was also significantly attenuated when NHERF-1 was silenced (Fig. 4B and C). This result supports a model in which NHERF-1 plays a central role in mediating Gq signalling pathways.

EGF-R can also be transactivated by Gqα via its action on membrane metaloproteases. This results in the liberation of heparin-binding EGF-like growth factor (HB-EGF) which in turn triggers the EGF signalling cascade [20,21]. Thus a reduction in the levels of NHERF-1 could alter cellular proliferation through both a direct effect on the EGF pro-proliferative pathway as well as indirectly reducing NT-R mediated EGF-R. Furthermore, a recent study has shown that the activation of MAPK/ERK by NT is dependent on the $[Ca^{2+}]_i$, hence silencing NHERF-1 and reducing the Ca^{2+} signal effectively shuts down this proliferative signalling pathway [36]. The activation of ERK is also dependent on protein kinase C and may involve NHERF-1 [37]. In conclusion, these data suggest a central role for NHERF-1 in the regulation of Gq-mediated signalling in epithelial cells. Overexpression of NHERF-1 as seen in many epithelial cell tumours may prolong normal proliferative signalling pathways resulting in increased proliferation. Further studies are required to determine the exact molecular basis for this role of NHERF-1 that may shed light on new strategies to attenuate epithelial tumour growth.

Acknowledgments

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