Uptake of Neisserial autotransporter lipoprotein (NalP) promotes an increase in human brain microvascular endothelial cell metabolic activity

Osman A. Dufailu, Jafar Mahdavi, Dlawer A. A. Ala'Aldeen[1], Karl G. Wooldridge and Neil J. Oldfield[*]

School of Life Sciences, University of Nottingham, Nottingham, UK

[1]Present address: Middle East Research Institute, Dream City, Erbil, Kurdistan Region of Iraq

[*]For correspondence: Centre for Biomolecular Sciences, University of Nottingham, University Park, Nottingham, NG7 2RD, UK. E: neil.oldfield@nottingham.ac.uk; T: (+44) 115 748 6122.

Abstract

Neisseria meningitidis is normally a human nasopharyngeal commensal but is also capable of causing life-threatening sepsis and meningitis. *N. meningitidis* secretes several virulence-associated proteins including Neisserial autotransporter lipoprotein (NalP), an immunogenic, type Va autotransporter harboring an S8-family serine endopeptidase domain. NalP has been previously characterized as a cell-surface maturation protease which processes other virulence-associated meningococcal surface proteins, and as a factor contributing to the survival of meningococci in human serum due to its ability to cleave complement factor C3. Here, recombinant NalP (rNalP) fragments were purified and used to investigate the interaction of NalP with host cells. Flow cytometry and confocal microscopy demonstrated binding and uptake of rNalP into different human cell types. High-resolution microscopy confirmed that internalized rNalP predominantly localized to the perinuclear region of cells. Abolition of rNalP protease activity using site-directed mutagenesis did not influence uptake or sub-cellular localization, but inactive rNalP ($rNalP^{S426A}$) was unable to induce an increase in human brain microvascular endothelial cell metabolic activity provoked by proteolytically-active rNalP. Our data suggests a more complex and multifaceted role for NalP in meningococcal pathogenesis than was previously understood which includes novel intra-host cell functions.

Highlights:

- Recombinant NalP is internalized by a variety of human cell types
- Internalized NalP is localized predominantly to the perinuclear region of cells
- Exposure to NalP provokes increases in cell metabolic activity
- Effects on cell metabolic activity are dependent on NalP proteolytic activity

Keywords: *Neisseria meningitidis*; autotransporter; NalP; cellular uptake; pathogenesis; proteolysis

1. Introduction

Neisseria meningitidis is an encapsulated Gram-negative diplococcus commonly carried in the human nasopharynx. Carriers usually remain asymptomatic, but rarely meningococci can reach the bloodstream which may lead to sepsis, and the cerebral-spinal fluid (CSF) which can result in meningitis [1]. *N. meningitidis* elaborates numerous virulence factors that facilitate colonization and virulence [2]. One important class, which are also found in many other Gram-negative bacteria, are the autotransporter (or type V-secreted) proteins [3]. These comprise an N-terminal signal peptide and C-terminal β-domain, which facilitate export of a central functional passenger domain across the Gram-negative inner and outer membranes, respectively. Following export, the passenger domain may be cleaved and released, either auto-catalytically or via a second protease, cleaved but remain non-covalently bound to the translocator domain, or remain uncleaved and displayed at the cell surface [4]. Autotransporter passenger domains undertake a range of virulence-associated functions including proteolytic, cytotoxic or adhesive activities [5].

The functional passenger domain of Neisserial autotransporter lipoprotein (NalP), also termed autotransporter serine protease A (AspA), harbors an S8-family peptidase (subtilisin) domain [6, 7]. Additionally, NalP expression is phase-variable via slipped strand mispairing, a process mediated by a poly-cytosine tract within the protein-coding sequence [7, 8]. Auto-catalytic proteolytic cleavage results in the release of a *ca.* 68-70 kDa passenger domain into the external environment [6, 7]. However, a proportion of the cleaved passenger domains remain, at least temporarily, anchored on the cell surface by an N-terminal lipid moiety [9]. This delayed or partial release facilitates the NalP-dependent proteolytic release of fragments

of other meningococcal surface proteins including: IgA1 protease; MspA (meningococcal serine protease A); App (adhesion and penetration protein); LbpB (lactoferrin-binding protein); and NHBA (Neisserial heparin binding antigen) [7, 10-14]. The consequences of the NalP-mediated release of meningococcal surface protein fragments on pathogenesis are beginning to be elucidated. For example, NalP-mediated release of NHBA abrogates extracellular DNA-mediated biofilm formation [15]. Furthermore, the NHBA-derived C2 fragment released following cleavage by NalP alters endothelial integrity by producing reactive oxygen species resulting in the internalization of the VE-cadherin adherens junction protein [16].

In addition to meningococcal targets, NalP has also been shown to cleave the host serum protein complement 3 (C3) [17]. NalP-mediated cleavage generates a functionally inactive C3a-like molecule, with the corresponding C3b-like fragment being rapidly inactivated by host serum factors resulting in the inhibition of C3b deposition on the bacterial surface and enhanced meningococcal serum resistance [17]. We hypothesized that, in addition to previously identified roles, NalP, like several other meningococcal autotransporter proteases [18-20], may also interact with host cells. Here we show uptake of NalP into various human cell types, and an increase in human brain microvascular endothelial cell metabolic activity which is dependent on NalP proteolytic activity.

2. Materials and Methods

2.1. Bacterial strains and culture conditions

Escherichia coli strain JM109 (Promega) was used for expression of recombinant His_6-tagged NalP passenger domain fragments. *E. coli* strain XL10-Gold (Agilent Technologies) was used for mutagenic plasmid construction. Strains were cultured at 37°C in Lysogeny broth (LB) or on LB agar supplemented with ampicillin (100 µg ml^{-1}). *N.*

meningitidis strain MC58 (ATCC® BAA335™) was cultured at 37°C, in an atmosphere of air plus 5% CO_2, on Columbia agar with chocolated horse blood (Oxoid).

2.2. Construction of plasmids encoding recombinant NalP proteins

Primers NalPF1 (CGCGGATCCCTGCATACCGGAGACTTTCC) and NalPR1 (CGCGGATCCGGCGAGACTGTTGAAGATGCG) were used to amplify a fragment of *ca.* 2 kb encoding amino acids ^{101}L to ^{784}A from *N. meningitidis* MC58 *nalP*. After digestion with BamHI, the PCR product was ligated into BamHI-digested pQE30 to yield plasmid pOD1. A single-nucleotide mutation (T to G) at nucleotide position 1276 of *nalP* was introduced into pOD1 using the QuikChange Lightning site-directed mutagenesis kit (Agilent Technologies) for expression of rNalP^{S426A}. The mutagenic reaction was undertaken following the manufacturer's instructions and utilized primers t1276g S426A (CCGATTCAAATTGCCGGAACAGCCTTTTCCGCACC) and t1276g_antisense (GGTGCGGAAAAGGCTGTTCCGGCAATTTGAATCGG) to yield pOD1^{S426A}.

2.3. Protein expression and purification

rNalP and rNalP^{S426A} were expressed in *E. coli* JM109 harboring pOD1, or pOD1^{S426A}, respectively, and purified under non-denaturing conditions. Briefly, *E. coli* JM109 strains were grown to OD_{600} 0.5 before being induced with 1 mM IPTG and incubated at 37°C for 3 h. Cells were harvested by centrifugation (4,200 × *g* for 10 min) and resuspended in 30 ml lysis buffer (50 mM Na_2PO_4, 300 mM NaCl and 20 mM imidazole, pH 7.4) followed by sonication using a MSE SoniPrep 150 sonicator for 8 cycles (30s on, 30s off) on ice. The cell lysate was centrifuged at 4,200 × *g* for 15 min and the cleared lysate loaded onto a 1 ml Nickel-charged HisTrap FF column connected to a ÄKTAprime plus liquid chromatography system (GE Healthcare Lifesciences) equilibrated with 10 column

volumes of wash buffer (50 mM Na_2PO_4, 300 mM NaCl and 40 mM imidazole, pH 7.4). Proteins were eluted by step elution with appropriate buffer (50 mM Na_2PO_4, 300 mM NaCl and 300 mM imidazole, pH 7.4). A HiTrap column pre-packed with five milliliters of Sephadex G-25 Superfine (GE Healthcare Lifesciences) equilibrated with 5 column volumes of phosphate buffered saline (PBS) was used for buffer exchange. His_6-tagged recombinant *E. coli* trigger factor protein (rTF), encoded by the plasmid pCold TF (Takara Bio), was purified as previously described [18]. Protein concentration was measured using the Pierce BCA protein assay kit (Thermo Fisher Scientific) following endotoxin removal using Pierce High Capacity Endotoxin removal spin columns (Thermo Fisher Scientific).

2.4. SDS-PAGE and immunoblot analysis

Proteins electrophoretically separated using polyacrylamide mini gels (Mini-Protean III; Bio-Rad) were stained with SimplyBlue™ SafeStain (Thermo Fisher Scientific) for 1 h. Alternatively proteins were transferred to nitrocellulose membranes and probed with rabbit polyclonal anti-NalP [6] or mouse anti-pentahistidine antibody (Qiagen) diluted 1:5,000 or 1:2,000, respectively, in blocking buffer (5% [w/v] non-fat dry milk, 0.1% [v/v] Tween 20 in 1 × PBS) and incubated for 2 h. Following washing in PBS with 0.1% Tween 20 (PBST), membranes were incubated for 2 h with goat anti-rabbit (or anti-mouse) IgG-alkaline phosphatase conjugate (Sigma) at a dilution of 1:30,000 in blocking solution. After washing with PBST, blots were developed using BCIP/NBT-Blue liquid substrate (Sigma).

2.5. Cell culture

All cells were maintained at 37°C in a humidified atmosphere of 5% CO_2. Cell culture reagents were purchased from ScienCell unless otherwise stated. Human brain microvascular endothelial cells (HBMECs-ATCC) were cultured on human fibronectin-coated flasks (BD

Biosciences) in basal endothelial cell medium, supplemented with 5% (v/v) heat-inactivated fetal bovine albumin (FBS), 1 × endothelial cell growth supplement and 1% (v/v) antibiotic / anti-mycotic solution. Dendritic cells were generated as previously [18], and subsequently grown in RPMI (Sigma) supplemented with 10% FBS and 1% antibiotic / anti-mycotic. Human brain (cerebral cortex) astrocytes (ScienCell) were cultured in astrocyte growth medium supplemented with 5% FBS, 1 × astrocyte growth supplement and 1% antibiotic / anti-mycotic. Hep-2 cells were cultured in DMEM (Sigma) supplemented with 10% FBS, 1 × non-essential amino acids (Sigma) and 1% antibiotic / anti-mycotic.

2.6. Flow cytometry

HBMECs were cultured on fibronectin-coated 24-well plates for 16 h from a seeding density of 5×10^4 cells ml^{-1}. rNalP was atto488-labelled using the Lightning-Link conjugation kit (Innova Biosciences) according to the manufacturer's instructions. Cells were treated with atto488 alone, atto488-labelled rNalP or unlabeled rNalP (250 nM final concentration dissolved in media) for 8 h. Cells were washed with PBS, fixed with 2% paraformaldehyde for 10 min, and washed again with PBS before being detached using trypsin-EDTA (Sigma). Harvested cells were washed twice with PBS and finally resuspended in 500 µl of PBS. Samples were run on a FC500 flow cytometer (Beckman Coulter). 100,000 events were recorded, and acquired data was analyzed using Kaluza v1.3 software.

2.7. Cell internalization assays

Proteins were Cy5-labelled using the Lightning-Link conjugation kit (Innova Biosciences) according to the manufacturer's instructions. Cells were seeded onto acid-etched 12 mm glass coverslips (pre-coated with 0.1% human fibronectin for HBMECs) in 24-well plates at a seeding density of 5×10^4 cells per well and incubated for 16 h. Cells were

treated with labelled rNalP or rTF (250 nM final concentration dissolved in media) for 45 min, 2 h, 4 h or 8 h. After treatment, cells were washed thrice with PBS, fixed in 4% paraformaldehyde for 10 min, and rinsed thrice in PBS. Cells were mounted using proLong Gold anti-fade with DAPI (Life Technologies) to stain cell nuclei. Confocal imaging was performed using a Zeiss LSM 700 confocal laser-scanning microscope using a Plan-Apochromat 63×/1.40 Oil DIC M27 objective with ZEN 2009 operating software, and equipped with lasers at 633 and 405 nm for excitation of Cy5 and DAPI, respectively. The images collected in the two channels were later merged and analyzed using Zeiss LSM and ImageJ software. Structured illumination microscopy (SIM) images were captured on a Zeiss Elyra PS.1 microscope using an Objective alpha Plan-Apochromat 100×/1.46 Oil DIC M27 objective with ZEN 2012 acquisition and processing software. Samples were excited using the 642 and 405 nm laser lines and fluorescence was detected using the LP 655 filter set. 3D reconstruction was carried out using ZEN 2012 Black software. 30,000 images were processed where single molecule events were identified with the peak intensity to noise value set to ten.

2.8. Human Complement 3 (C3) protein cleavage assay

This was performed as previously described [17] with minor modifications. Briefly, 40 nM recombinant protein was mixed with 250 ng human complement 3 (C3; Sigma) in PBS and incubated at 37°C with shaking for 16 h. C3 cleavage was determined by immunoblot analysis using goat anti-human C3 antibody (diluted 1:5000; Sigma) and rabbit anti-goat IgG alkaline phosphatase conjugate (diluted 1:30000; Sigma).

2.9. XTT assay

HBMECs were seeded in 96-well plates (1×10^4 cells $well^{-1}$) and grown overnight as outlined in section 2.5. Cells were supplied with serum-free media and incubated at 37°C with 5% CO_2 for 1 h before the addition of recombinant protein (40 nM final concentration) and XTT detection solution (Cell Signaling Technologies). Cells were incubated for 37°C with 5% CO_2 for 750 min and absorbance measurements (450 nm) taken using a Spark microplate plate reader. Each test was performed in triplicate wells and on a least three independent occasions. GraphPad Prism v7 was used to analyze the data using two-way ANOVA and Tukey's multiple comparison test. A p value < 0.05 was considered statistically significant.

2.10. Histone clipping

Recombinant core histones (New England Biolabs; 200 μg ml^{-1} final concentration) and purified recombinant protein (40nM final concentration) were mixed in a total volume of 40 μl PBS, and incubated at 37°C for 16 h. Reactions were stopped by the addition of 5 × sample buffer (0.62 M Tris-HCl [pH 6.8], 5% SDS, 25% glycerol, 12.5% β-mercaptoethanol, 0.25 M DTT, 0.002% bromophenol blue), followed by immediate boiling for 5 min. Aliquots of cleavage products were subjected to SDS-PAGE, and gels strained with SimplyBlue™ SafeStain (Thermo Fisher Scientific).

3. Results

3.1. NalP is internalized by human cells

The passenger domain of the group B meningococcal strain MC58 NalP protein was expressed in *E. coli* and purified under non-denaturing conditions to yield rNalP. Co-incubation with human brain microvascular endothelial cells (HBMECs) followed by

examination by flow cytometry showing a clear shift in fluorescence signal in HBMECs treated with fluorescently labelled rNalP compared with HBMECs treated with label alone or with unlabeled rNalP, suggesting the direct interaction of rNalP with host cells (Fig. 1). Confocal laser scanning microscopy was subsequently used to visualize rNalP following co-incubation with HBMECs (Fig. 2). From 2 h co-incubation, internalized rNalP was primarily apparent in crescent-like perinuclear accumulations (Fig. 2). In contrast, an unrelated His_6-tagged recombinant protein (*E. coli* trigger factor; rTF), which was purified using the same methodology as rNalP, was not internalized, demonstrating cellular specificity for rNalP uptake (Fig. 2). Localization of internalized rNalP predominantly to the perinuclear region of HBMECs was confirmed using high-resolution microscopy (Fig. S1). Examination by confocal microscopy of other human cell types, including dendritic cells (DCs), epithelial cells (Hep-2) and neural cells (astrocytes) showed that rNalP was also taken up by these cell types and similarly localized to the cytoplasmic and/or perinuclear regions (Fig. S2).

3.2. Uptake of proteolytically-active rNalP promotes an increase in HBMEC metabolic activity

Site-directed mutagenesis was used to replace the catalytic serine residue of rNalP with alanine to generate $rNalP^{S426A}$. The abolition of proteolytic activity was confirmed by the inability of $rNalP^{S426A}$ to generate a *ca.* 100-kDa α-chain fragment following incubation with the NalP target human complement factor C3 [17] (Fig. S3). Examination by confocal microscopy showed no apparent differences in the localization of $rNalP^{S426A}$ or rNalP in HBMECs after co-incubation of cells with these proteins, showing that rNalP proteolytic activity was not required for uptake or subsequent sub-cellular localization (Fig. 3).

To examine the cellular response to NalP, HBMECs were monitored post-exposure to rNalP or $rNalP^{S426A}$ using the XTT assay, which measures the ability of metabolically active

cells to reduce tetrazolium salt. From 270 min onwards, cells exposed to rNalP exhibited a statistically significant increase in absorbance compared to untreated cells (Fig. 4). In contrast, at no time point did HBMECs exposed to rNalP^{S426A} or rTF show significantly different absorbance to untreated cells. As expected, cells exposed to the known apoptosis-inducing agent staurosporine, which would be expected to reduce cellular metabolic activity, had a significantly reduced absorbance evident from 120 min post-exposure. These data show that rNalP can induce an increase in HBMEC metabolic activity and this effect is mediated via the proteolytic activity of rNalP.

We previously showed that the meningococcal autotransporter proteins App and MspA are capable of clipping the histone H3 [18]. To investigate potential cellular targets for rNalP proteolytic activity, we examined the ability of rNalP and rNalP^{S426A} to cleave human histones (H2A, H2B, H3.1 and H4) but the absence of rNalP-specific cleavage products confirmed a lack of histone clipping activity (Fig. S4).

4. Discussion

NalP is an autotransporter protease which cleaves itself, and several other meningococcal proteins, on the outer surface of the bacterium [7, 10-14]. Additionally, NalP cleaves human C3, which contributes to meningococcal survival in human serum [17]. Here we provide evidence that NalP is internalized by human cells and induces alterations in host cell biology. This is reminiscent of findings from previous studies on the IgA1P, App and MspA meningococcal serine protease autotransporters [18, 20, 21]. Following cellular uptake and trafficking, IgA1P cleaves the p65/RelA component of NF-kB in the nucleus, thus silencing the expression of several NF-kB-responsive genes, ultimately leading to sustained activation of c-Jun N-terminal kinase and apoptosis [20]. App and MspA are also internalized and induce apoptosis but, in contrast to IgA1P, MspA/App-mediated apoptosis occurs via

cleavage of histones [18]. The receptor of IgA1P uptake is unknown, whilst uptake of App and MspA requires the cooperative activity of the mannose and transferrin receptors [18]. The wide range of cell types able to mediate NalP internalization (including immune, epithelial, endothelial and neural cells) suggests shared receptor and internalization machineries, but this will require further experimentation. Additional work is also required to define the region(s) of NalP which are involved, although our data shows that uptake is not dependent on the proteolytic ability of NalP.

The contribution of NalP to pathogenesis remains incompletely understood, in part because of its low abundance in culture supernatants, which has impeded the purification of experimentally useful amounts of active NalP directly from meningococcal cultures. Consequently, previous studies have utilized *E. coli*-derived recombinant NalP fragments purified under denaturing conditions (*i.e.* non-proteolytically active) or concentrated secretome preparations derived from *N. meningitidis*, which although containing active NalP, lacked purity. Furthermore, wild-type and *nalP* mutant-derived secretomes are difficult to compare directly given the complex alterations in secreted protein profile that are NalP-expression dependent [7, 9-14, 20]. The ability of our rNalP preparation, which was purified under non-denaturing conditions, to cleave human C3, confirmed the findings of Del Tordello *et al.*, [17], demonstrated that the recombinant protein was catalytically active, and importantly provided the opportunity to specifically address the influence of NalP and its proteolytic activity in inducing changes in host cell biology. This was determined using the XTT assay – an assay based on the ability of dehydrogenase enzymes produced by metabolically-active cells to reduce XTT to an orange formazan dye [22]. Significant alterations in the ability to reduce XTT usually result from changes in cell viability following exposure to a treatment. Additional work is required to investigate if the positive effect mediated by rNalP occurs in other human cell types, whether it results from increased

proliferation, and to define the mechanism by which the effect occurs. Our data confirm, however, that the observed effect is not mediated via NalP-mediated histone clipping.

In summary, the use of proteolytically-active recombinant NalP has generated new insights into the multifaceted role of NalP in the complex interaction between the meningococcus and its obligate host by revealing that NalP is internalized by host cells and that NalP proteolytic activity results in an increase in cell metabolic activity.

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Conflicts of interest

The authors declare that there are no conflicts of interest.

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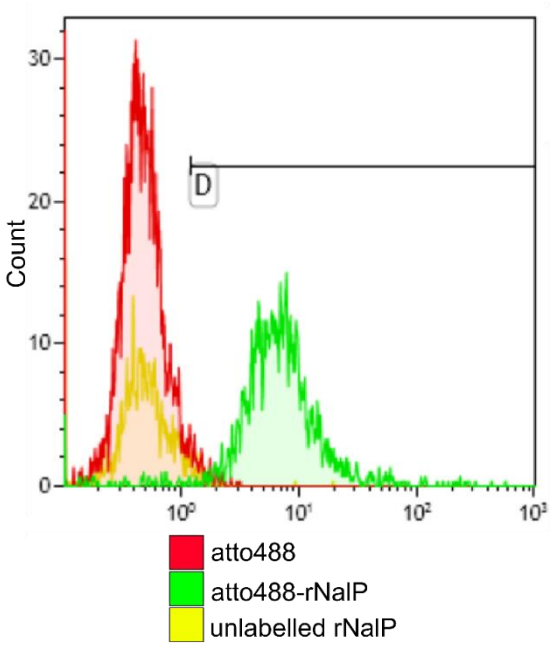

Fig. 1. Flow cytometric analysis showing the binding of rNalP to human brain microvascular endothelial cells (HBMECs). HBMECs treated with atto488 alone (red) were compared with HBMECs treated with atto488-rNalP (green) and unlabeled rNalP (yellow). Data was acquired on a Beckman Coulter FC500 flow cytometer and is displayed as a histogram. The histogram area in D represents the population of fluorescently labelled HBMECs.

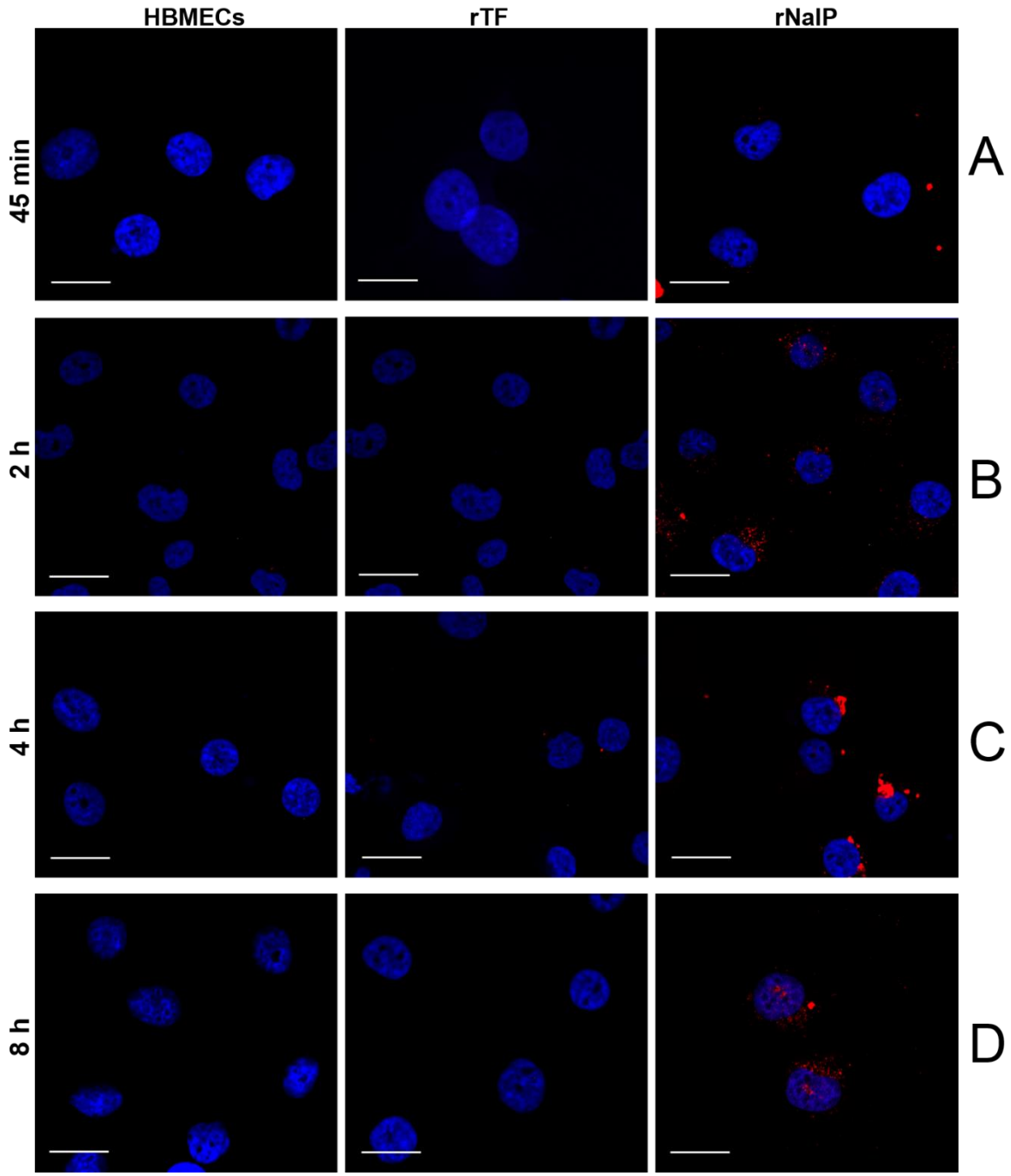

Fig. 2. Cellular uptake of rNalP into human brain microvascular endothelial cells (HBMECs). HBMECs were left untreated or co-incubated with Cy5-labelled rTF or rNalP for 45 min (A), 2 h (B), 4 h (C) or 8 h (D). Cell nuclei were stained with DAPI. Cells were washed and fixed before analysis by confocal laser microscopy. All images were scanned at a resolution of 1024 × 1024 pixels, using the same laser and gains settings. The cells are representative of cells observed from three independent experiments. Scale bars = 20 µm.

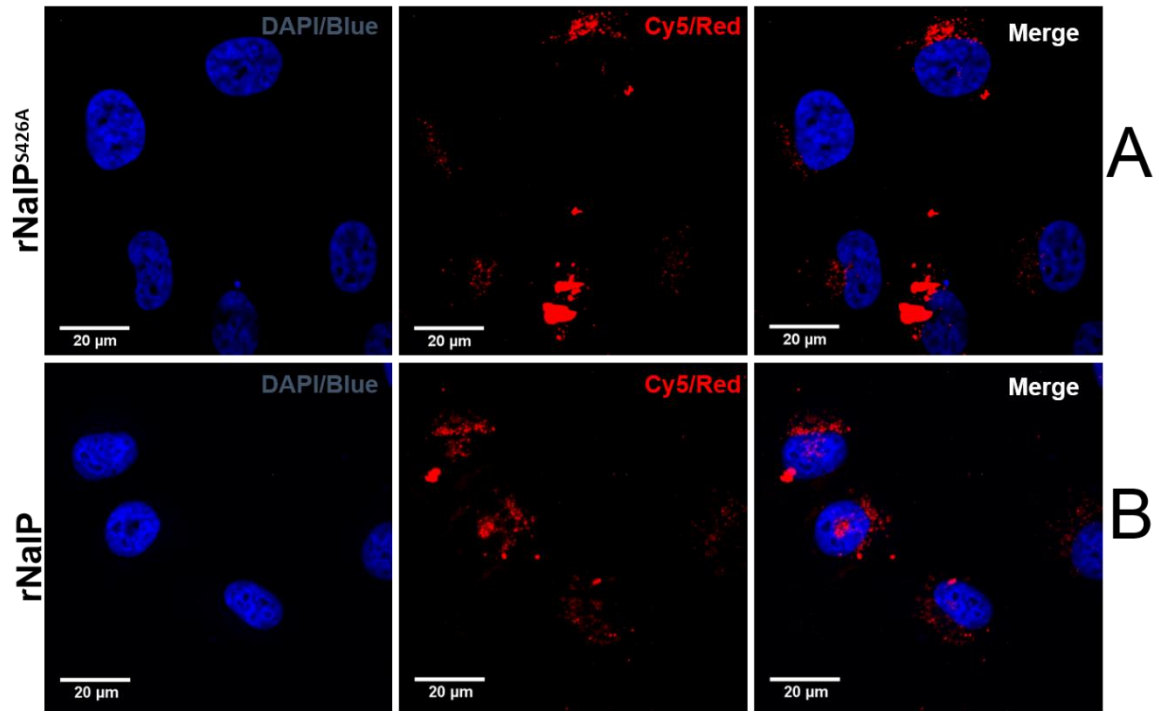

Fig. 3. rNalP proteolytic activity is not required for cellular uptake and perinuclear localization by HBMECs. HBMECs were treated with Cy5-labelled rNalP^{S426A} (A) or rNalP (B) for 8 h. Cell nuclei were stained with DAPI, washed and fixed before scanning using confocal laser microscope. All images were scanned at a resolution of 1024 × 1024 pixels, using the same laser and gains settings. The cells are representative of cells observed from multiple experiments. Scale bars = 20 µm.

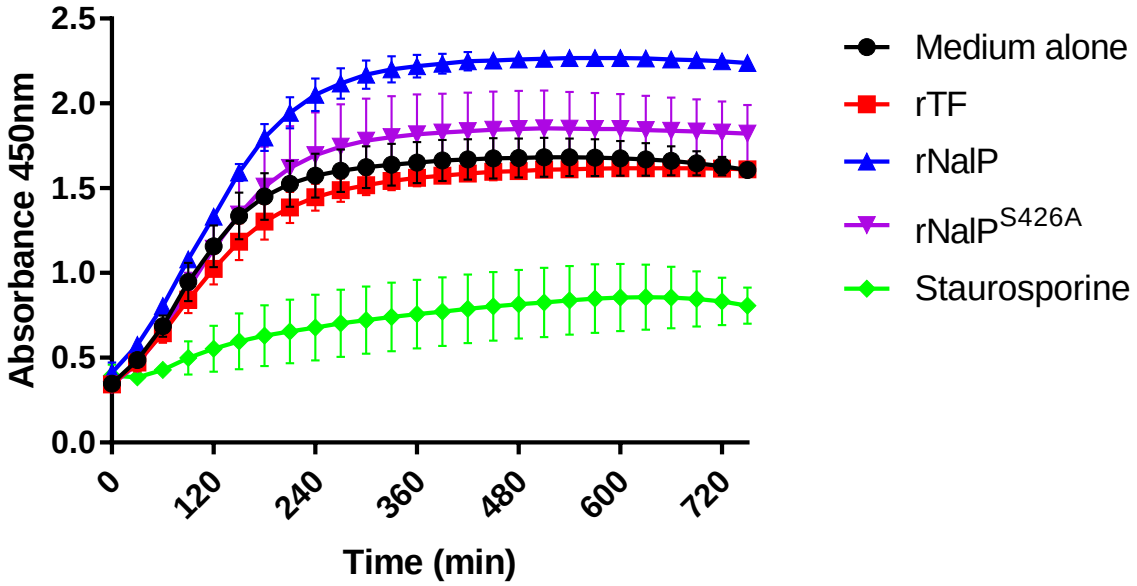

Fig. 4. Exposure of HBMECs to rNalP induces an increase in cell metabolic activity. HBMECs were treated with medium alone, rTF, rNalP, rNalP^{S426A} or staurosporine in triplicate. Cell metabolic activity was assessed using the XTT assay kit via absorbance readings at 450 nm over 750 min. Values shown are the mean ± SE from three independent experiments and were analyzed by two-way ANOVA and Tukey's multiple comparison test. Significant increases in absorbance were induced by rNalP ($p<0.05$ from 270 min; $p<0.005$ from 600 min). rNalP^{S426A} and rTF induced no significant difference compared to media alone at any time point, whilst staurosporine induced a significant reduction in absorbance compared to treatment with media alone ($p<0.01$ from 120 min; $p<0.001$ from 150 min; $p<0.0001$ from 180 min).