Biochimica et Biophysica Acta 1788 (2009) 442–449

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamem

Apolipoprotein E peptide-modified colloidal carriers: The design determines the mechanism of uptake in vascular endothelial cells

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ARTICLE INFO

Article history:
Received 27 June 2008
Received in revised form 23 October 2008
Accepted 28 November 2008
Available online 14 December 2008

Keywords:
Peptide micelles
Liposome
Endocytosis
Inhibitor
Cell-penetrating peptide

ABSTRACT

Supramolecular structures, particularly micelles and liposomes equipped with uptake-mediating address compounds, have attracted much attention as pharmaceutical formulations. Their development requires an understanding of the mechanism by which the carrier systems interact with and translocate into the target cells. We developed an apolipoprotein E-derived peptide, called A2, that efficiently translocates across cell membranes. Upon coupling of two palmitoyl chains (P2), the highly cationic sequence acquires detergent-like properties such as a strong tendency to self-associate and the ability to integrate into lipid bilayers. Confocal laser scanning microscopy and fluorescence activated cell sorting were used to compare the internalization of the fluorescence-labeled monomeric A2 with the uptake of the colloidal P2A2 micelles and P2A2-tagged liposomes into endothelial cells of blood vessels. Specific inhibitors of endocytosis were used to identify the underlying mechanisms. b.End3 and BAEC cells as example of endothelial cells of small capillaries and large vascular vessels, respectively, were examined. The uptake of monomeric A2 was characterized by poor cellular selectivity. A2 was efficiently internalized into both cell lines via at least two different mechanisms. Besides an endocytotic uptake route, a second passive pathway exists, that leads to a rapid distribution of A2 within the cytoplasm. Also liposomes tagged with P2A2 were non-selectively internalized into both b.End3 and BAEC cells. Their nonselective uptake was mediated by clathrin- and caveolin-independent endocytosis. In contrast, micellar P2A2 entered b.End3 cells via clathrin-mediated endocytosis, while no uptake of P2A2 into BAEC cells was observed. In conclusion, the specific clathrin-mediated uptake mode of P2A2 micelles might provide the basis for a blood brain barrier-specific targeting.

1. Introduction

A prerequisite for successful application of all bioactive agents is the access to their site of action. For agents with intracellular targets the transmembrane transport can constitute a major obstacle. Especially large, hydrophilic molecules tend to have poor pharmacokinetics and cannot cross the plasma membrane [1]. One approach to overcome these limitations is the incorporation into or attachment to molecular carriers, e.g. liposomes or micelles [2]. Their properties such as vesicle size, surface charge density, and sterical stability can easily be adapted to meet variable requirements and thus to increase their range of application [3]. However, the potential advantages of carrier administration are often limited by a low specificity. A favorable method to overcome this problem is the derivatization of the carrier with target-recognizing and uptake-mediating ligands [4]. One possibility is the application of so called cell-penetrating peptides (CPPs), which can facilitate the transport of attached cargos across the cellular barrier. So far the mechanism of CPP internalization is not well understood and reports are often conflicting [5]. Earlier studies proposed a receptor- and energy-independent mechanism [6,7] whereas more recently, also active endocytotic mechanisms have been proposed [8,9]. In some cases, the internalization depends on strong ionic interactions between the positively charged peptides and negatively charged constituents of the cell surface such as heparan sulfate proteoglycanes (HSPGs) [10,11]. Cargos delivered by CPPs are very diverse in size and structure. They range from small molecules like biotin or doxorubicin [12] and peptides [13] up to large proteins [14] and liposomes [15,16]. It is reasonable to assume an influence of the cargo on the internalization process. For the TAT peptide, derived from the transactivator of transcription protein of HIV-1 [17], transduction as main entry process has been reported whereas endocytosis becomes predominant with increasing size of attached cargos [18]. Attempts to characterize such relations in detail are scarce so far.

A peptide exhibiting certain characteristics of CPPs is A2 (LRK LRK RLLR)$_2$, a tandem dimer derived from the low density lipoprotein-receptor (LDLr) binding-domain of apolipoprotein E (141–150)$_2$. The sequence comprises binding sites for the LDLr [19,20] as well as for cell-surface HSPGs [21,22]. Covalently coupled, A2 mediates an efficient internalization of sterically stabilized liposomes into primary rat brain capillary endothelial cells [16]. N-terminal dipalmitoylation (P2) confers surfactant-like properties upon the cationic A2. The

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0005-2736/$ – see front matter
doi:10.1016/j.bbamem.2008.11.025

lipopeptide forms stable micelles and rapidly incorporates into liposomes [23,24]. Both colloidal systems efficiently translocate across the membrane of brain capillary endothelial cells. Studies with P2A2-tagged liposomes containing fluorescein isothiocyanate-dextran as fluorescent probe in the aqueous liposomal lumen suggest that the liposomes are internalized intact [24,25].

Different pinocytotic mechanisms mediate the entry of particulate structures into mammalian cells [26]. Large systems can be internalized by macropinocytosis or membrane ruffling [26]. The well characterized clathrin-mediated endocytosis (CME) is crucial for many cellular processes, among others the uptake of essential nutrients or transferrin and LDL via receptor binding [27]. Another endocytotic process is based on caveolae which are abundant in endothelial cells where they constitute 10–20% of the cell surface. The uptake of serum albumins, for instance, is mediated by receptors localized in caveolae [28]. Another pinocytic mechanism is merely described as non-clathrin and non-caveolin-dependent. So far this mode of cellular uptake is poorly understood and most likely more than one mechanism is involved. The various uptake modes result in endocytotic vesicles with different properties [26]. As the mode of transmembrane transport determines the intracellular fate of molecules and colloidal systems [29,30], our interest has been focused upon the elucidation of the mechanism of uptake of the different A2 formulations. Different pharmacological inhibitors were used to analyse the endocytotic pathways. As endocytosis is based on the activity of the cytoskeleton, application of the F-actin-depolymerizing drug cytochalasin D results in a blockade of all endocytotic processes [31,32]. Chlorpromazine prevents the formation of clathrin lattices at the plasma membrane and thus efficiently inhibits CME [33]. Caveolar-mediated endocytosis, which is characterized by the assembly of caveolin in the plasma membrane, is blocked by the cholesterol-complexing drug nystatin [34].

Endothelial cells of the blood vessels constitute the first contact area of intravenously applied drug formulations. To assess the transmembrane transport properties of cells lining blood capillaries and large vessels, we compared the uptake of the monomeric A2 peptide, small P2A2 micelles and P2A2-tagged large liposomes into brain capillary endothelial cells (b.End3) [35,36] and aortic endothelial cells (BAEC) [37].

2. Materials and methods

2.1. Materials

Protected amino acids were purchased from GLS (Shanghai, China) or Novabiochem (Darmstadt, Germany). Palmitic acid, other chemicals, and solvents used for peptide synthesis were from Fluka (Buchs, Switzerland). The lipids 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) and 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-[lissamine rhodamine B sulfonyl] (ammonium salt) (Rhod-DPPE) were obtained from Avanti Polar Lipids (Alabaster, USA). Calcein was from Fluka (Deisenhofen, Germany). The tissue-culture media and fluorescein-conjugated transferrin were from Gibco®/Invitrogen™ (Carlsbad, USA). (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl)tetrazolium bromide (MTT) was purchased from Sigma (Munich, Germany).

2.2. Peptide synthesis and characterization

The peptides were synthesized on solid phase (TentaGel-S-RAM; Rapp Polymere, Tuebingen, Germany) using an ABI 433A automatic peptide synthesizer (Applied Biosystems, Foster City, USA) following the standard Fmoc (9-fluorenylmethoxycarbonyl) chemistry protocol [38,39]. The tandem repeat A2 $(141–150)_2$ was N-terminally modified with carboxyfluorescein (f). The carboxyfluorescein labeled peptide P2fA2 was synthesized using a Fmoc-lysine(Dde) derivative (Nα-(9-fluorenylmethoxycarbonyl)-Nε-1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)ethyl-L-lysine) and a Fmoc-lysine(Fmoc) derivative (Nα-(9-fluorenylmethoxy-carbonyl)-Nε-(9-fluorenylmethoxycarbonyl)-L-lysine) at the N-terminus [16,23]. Acylation of A2 and fA2 was performed overnight with a 10-fold excess of palmitic acid activated with diisopropylcarbodiimide/N-hydroxybenzotriazole. Subsequently the Dde group was cleaved off with 2% hydrazine in dimethyl formamide for 10 min and carboxyfluorescein was coupled with diisopropylcarbodiimide/N-hydroxybenzotriazole at a 4-fold excess overnight. Cleavage from the resin with 10% phenol in trifluoroacetic acid (TFA) for 3 h gave the C-terminally amidated peptides.

Purification was carried out by preparative reverse phase high-performance liquid chromatography (RP-HPLC) using a LC-8A system (Shimadzu, Kyoto, Japan) with a PLRP-S 10 μm column (Polymer Laboratories, Amherst, USA). A purity better than 95% was achieved as determined by analytical RP-HPLC on a LC-2010C system (Shimadzu, Kyoto, Japan) using a Prontosil 300-5-C18 column (Bischoff, Leonberg, Germany).

The peptides were further characterized by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF) on a Voyager-DESTR (Applied Biosystems, Foster City, USA) to confirm the expected masses.

2.3. Particle preparation

Micelles instantly form by self-association [23] when lyophilized P2A2/P2fA2 is dissolved in Dulbecco's phosphate buffered saline, pH 7.4, supplemented with 1 g/l D-glucose (DPBSG).

Large and small unilamellar vesicles (LUVs and SUVs, respectively) were prepared as described previously [24]. In short, dry POPC films were suspended in DPBSG by vortexing and SUVs and LUVs were produced by subsequent sonification or 35-times extrusion through two stacked polycarbonate filters with 100 nm pores using a MiniExtruder (Avestin, Ottawa, Canada), respectively. For flow cytometric measurements POPC-LUVs with 0.1% Rhod-DPPE were prepared. P2fA2- or P2A2-liposome complexes were prepared by mixing both components at appropriate concentrations.

2.4. Particle characterization

The liposome size was checked by dynamic light scattering (DLS) on a N4 Plus particle sizer (Beckman Coulter, Fullerton, USA).

Liposomal integrity was determined in a dye release experiment. Aliquots of POPC-LUV suspensions with internally trapped calcein at self-quenching concentration (80 mM in 10 mM Tris, 0.1 mM EDTA, pH 7.4) were injected into cuvettes containing 2.5 ml stirred peptide solutions (varying peptide concentrations in 10 mM Tris, 154 mM NaCl, 0.1 mM EDTA, pH 7.4). The final lipid concentration was 25 μM.

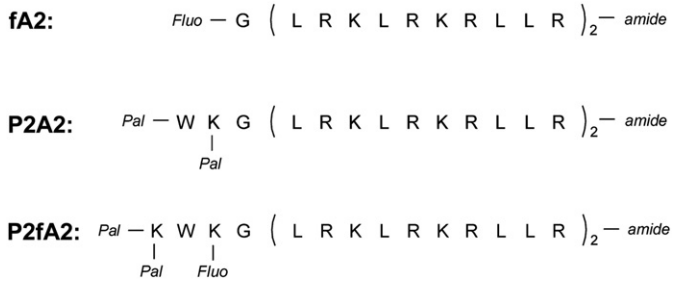

Fig. 1. Sequences of apolipoprotein E peptides in one-letter amino acid code. All peptides are C-terminally amidated. fA2 (MW: 3098.2 g/mol) is labeled with carboxyfluorescein at the N-terminus via a glycine linker. P2A2 (MW: 3530.9 g/mol) is equipped with a short N-terminal linker sequence and palmitoylated at the N-terminal tryptophan and the side chain of lysine at position 2. P2fA2 (MW: 4019.3 g/mol) is equipped with a short N-terminal linker sequence. The two palmitoyl chains are linked to the N-terminal lysine and carboxyfluorescein is coupled to the side chain of lysine at position 3.

Calcein release from liposomes was monitored fluorimetrically by measuring the increase in fluorescence intensity (λ_{ex}=490 nm, λ_{em}=520 nm) after 5 min at room temperature on a LS 50B spectrofluorimeter (Perkin Elmer, Waltham, USA). The fluorescence intensity corresponding to 0% and 100% release was measured in the absence of peptide and after addition of Triton X-100 (100 µl, 10% v/v in water), respectively. The $C_{l/p}$ of half maximal dye release was determined from dose–response curves by a sigmoid fit using Sigma Plot (Systat Software, San Jose, USA).

Fluorescence spectra of the carboxyfluorescein-labeled peptides fA2, P2fA2, and P2fA2-LUV complexes(λ_{ex}=488 nm, λ_{em}=500–600 nm) in DPBS were recorded using a FP-6500 fluorescence spectrometer (Jasco, Tokyo, Japan).

2.5. Cell culture

Immortalized mouse brain endothelial cells (b.End3) (ECACC 96091929) [35,36] and bovine aortic endothelial cells (BAEC) [37] were cultured in Earle's minimal essential medium (E-MEM) supplemented with 10% fetal bovine serum (FBS), 2 mM N-acetyl-L-alanyl-L-glutamine, 100 U/ml penicillin, and 100 U/ml streptomycin at 37 °C in a humidified atmosphere of 5% CO_2.

2.6. Toxicity test

Cell viability was determined using the MTT method [40,41]. A total of 2.5×10^4 cells per well were seeded 24 h prior to the experiment in a 96-well plate. The cells were exposed to increasing concentrations of A2 peptides or A2 peptide-labeled liposomes in DPBSG for 30 min at 37 °C. Cells treated with DPBSG referred to as 100% viable. Afterwards the cells were incubated in 180 µl E-MEM and 20 µl of 5 mg/ml 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) in DPBS per well for 4 h at 37 °C. The medium was removed and 100 µl dimethylsulfoxide was added per well. The absorption at 550 nm was measured using a Safire microplate reader (Tecan, Zurich, Switzerland). Cell viability was determined by normalizing the signals to 100% and plotted against peptide concentrations.

2.7. Confocal-laser-scanning microscopy (CLSM)

CLSM was employed in order to monitor the uptake of the A2 formulations and to determine their intracellular location. Cells were plated on poly-L-lysine-coated cover slips (diameter 30 mm) positioned in 35 mm plastic culture dishes and cultured for one day. The exponentially growing cells were washed with DPBSG and exposed to different A2-formulations and/or transferrin in DPBSG for 1 h. After washing two times with ice-cold DPBSG, cells were kept on ice until CLSM pictures were taken.

The cell vitality was checked by trypanblue exculsion. One drop of 0.5% trypanblue in PBS was put onto the surface of the microscopic sample.

CLSM pictures were taken using an LSM 510 inverted confocal laser scanning microscope equipped with a Plan-Neofluar 100×/1.3 oil objective (Carl Zeiss, Oberkochen, Germany). For carboxyfluorescein labeled samples, excitation was performed using a 200 mW argon laser (488 nm) at 10% intensity and emission was registered using a BP505–530 bandpass filter. Trypanblue was excited at 633 nm with a 15 mW helium-neon laser (633 nm) at 30% intensity and emission was recorded using a LP650 cutoff filter. The pinhole was set to 300 µm. Image acquisition was done sequentially to minimize cross-talk between the fluorophores.

2.8. Flow cytometry (FACS)

To investigate the internalization of different A2 formulations, exponentially growing adherent cells were washed with DPBSG at 37 °C and then incubated with peptides for 1 h at 37 °C (see figure legends for concentrations). In order to reduce the fluorescence of surface-bound peptide and detach the cells, they were washed two times with ice cold DPBSG and incubated for 5 min with 1 ml of 0.1% pronase E in DPBS with 0.5 mM EDTA at 4 °C. The reaction was stopped by adding 0.7 ml of 1% BSA in DPBS. The cell suspension was centrifuged for 4 min at 4500 ×g and 4 °C. The cell pellet was kept on ice until the measurement was performed. Immediately before the analysis the cells were resuspended in ice cold 0.5–1.0 ml 1% BSA in DPBS (depending on the cell density) and 8 µl 0.5% trypanblue per 0.5 ml volume was added to further reduce extra cellular fluorescence [42].

To examine the mechanism of internalization, a pre-incubation step with endocytosis inhibitors was introduced followed by co-incubation of the samples and the respective inhibitor. Pre incubation and uptake studies were performed for 1 h at 37 °C with 2 mg/ml cytochalasin D, 10 mg/ml chlorpromazine, or 25 mg/ml nystatin, respectively, in DPBSG.

Fluorescence analysis was performed using a FACSCalibur fluorescence-activated cell sorter (BD Biosciences, Franklin Lakes, USA) using a 530/30 nm bandpass filter (FL1) for carboxyfluorescein/ fluorescein with 500 V detector voltage for fA2 and 550 V for P2fA2 and fluorescein-conjugated transferrin incubated samples. A 585/42 nm bandpass filter (FL2) with 550 V detector voltage was used for rhodamine labeled samples. Viable cells for analysis were gated by dot plots. The respective scatter characteristics were determined before by backward-gating of propidium iodide negative cells. A minimum of 10,000 events per sample was analyzed. Each experiment was performed at least twice in duplicates or triplicates.

3. Results

3.1. Characterization of A2, micellar, and liposomal peptide complexes

The apoE-derived peptides used in this study are shown in Fig. 1. The biophysical properties of the peptides and P2A2-liposome complexes have been described previously [23,24]. Monomeric A2 shows only weak interaction with neutral POPC lipid bilayers [19,24,43]. The dipalmitoylated peptide P2A2 forms micelles in aqueous solution with a Stokes radius of 5.3 nm. The molecular mass of 68,000 g/mol, determined by analytical ultracentrifugation, corresponds to 19 P2A2 molecules per micelle [23]. The peptide-surface density is 5.38×10^{-4} P2A2/Å^2. At a molar lipid to peptide ratio $C_{l/p}$ of 1000 binding of P2A2 and P2fA2 to preformed POPC LUVs with a diameter of 100 nm had no significant influence upon the size distribution. Dye release experiments with P2A2 and liposomal trapped calcein yielded a half-maximal dye release at a $C_{l/p}$ of 450 (Fig. 2A). Thus, at a $C_{l/p}$ of 1000 the liposomal integrity was not affected. The number of surface-located P2A2 molecules was calculated to be 100, corresponding to a peptide-surface density of 3.18×10^{-5} P2A2/Å^2.

In Fig. 2B the carboxyfluorescein fluorescence spectra of monomeric fA2, micellar P2fA2, and liposomal-bound P2fA2 recorded at identical concentration are presented. Micellar P2fA2 showed a strong decrease in intensity to about 33% compared to fA2. This reflects presumably a self-quenching effect of carboxyfluorescein in the micellar structure. Binding to POPC-SUVs at a $C_{l/p}$ of 1000 results in a partial restoration of fluorescence, reaching 80% of the intensity observed for monomeric fA2.

3.2. Toxicity

The viability of both b.End3 and BAEC cells in the presence of monomeric fA2, micellar P2fA2, and liposomes tagged with P2A2 at a $C_{l/p}$ of 1000 is presented in Fig. 3. Pure POPC-liposomes show minor toxicity, as the cell viability remains over 80% at lipid concentrations up to 7.5 mM (data not shown).

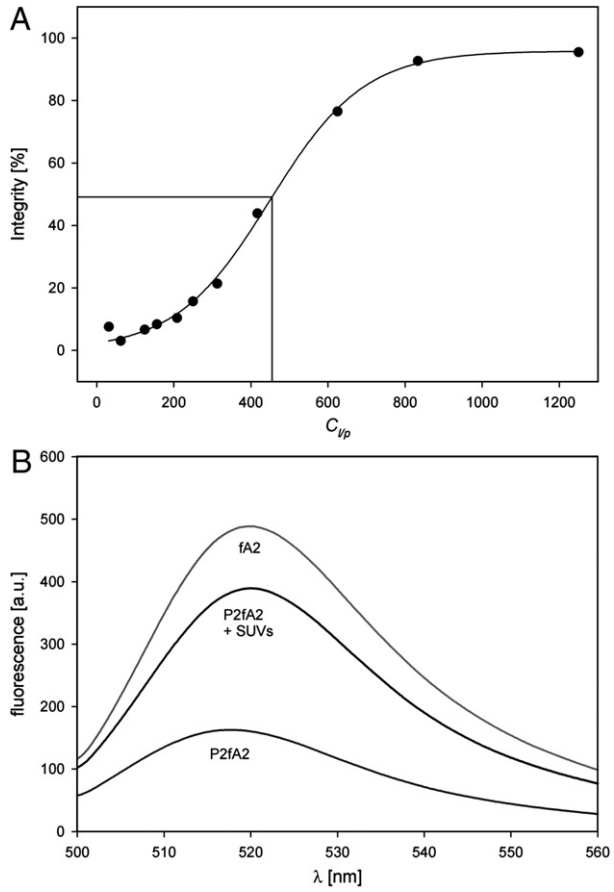

Fig. 2. (A) Liposomal integrity was determined by monitoring the increase in fluorescence after dilution of POPC-LUVs with internally trapped calcein at self-quenching concentration in stirred P2A2 solutions. Fluorescence intensity was measured at λ_{ex}=490 nm and λ_{em}=520 nm after 5 min at room temperature. The fluorescence intensity corresponding to 0% was measured in absence of peptide and 100% release was determined after the addition of Triton X-100 for each sample. Liposomal integrity [%] was calculated by normalizing the resulting fluorescence intensity to the 100% signal and plotted against the corresponding lipid to peptide ratios ($C_{l/p}$). The $C_{l/p}$ of half-maximal dye release was determined from dose–response curves by sigmoid fitting. (B) Fluorescence spectra of fA2, P2fA2, and P2fA2 in presence of POPC SUVs ($C_{l/p}$=1000) in DPBS. Peptide concentration is 0.5 μM for all peptides. The fluorescence was excitated at 488 nm.

The toxic activity of the different A2 formulations on b.End3 cells is low up to 10 μM peptide concentrations (Fig. 3A). At 1 μM fA2 and liposomal bound P2fA2 the viability of b.End3 cells is 90%, at 1 μM micellar P2fA2 it is still 75%.

BAEC cells show a more distinct susceptibility to the toxic effect of fA2 (Fig. 3B). Up to 1 μM fA2 and micellar P2fA2 nearly no toxic effect was observed. Above 1 μM a strong decrease in cell survival is seen, resulting in 20% viability at 10 μM peptide concentration. Liposomal bound P2fA2 has a considerably higher concentration-dependent toxicity. At 1 and 10 μM the cell viability was found to be 50% and 20%, respectively.

3.3. Cellular uptake (CLSM)

Monomeric fA2 is efficiently internalized into b.End3 and BAEC cells. CLSM studies with fA2 at 37 °C reveal a vesicular intracellular fluorescence superimposed by an evenly distributed cytosolic fluorescence in both cell lines (Fig. 4A). The contribution of both fluorescence patterns to the overall fluorescence is highly variable. Additionally, as the cytosolic fluorescence is still observed at 4 °C, while active uptake mechanisms are blocked or at least considerably decreased, a second, non-active mechanism must be postulated for the internalization of fA2. The integrity of the plasma membrane was untouched as assayed by trypanblue exclusion.

Also liposomes tagged with P2fA2 are rapidly internalized into both b.End3 and BAEC cells at 37 °C into cytoplasmic vesicles (Fig. 4B). At 4 °C no intracellular fluorescence was observed. Both observations suggest endocytosis as internalization mechanism.

Cell incubation with micellar P2fA2 at 37 °C lead to a strong vesicular fluorescence in b.End3 cells whereas the uptake into BAEC was drastically reduced (Fig. 4C). As the uptake into b.End 3 cells is blocked at low temperature, an endocytotic process is suggested. The striking difference in the internalization of P2fA2 micelles into brain capillary and aortic endothelial cells led to the assumption that different uptake mechanisms were addressed.

3.4. Uptake mechanism (FACS)

Fluorescent activated cell sorting (FACS) was used in order to quantify the inhibitory effect of cytochalasin D, chlorpromazine, and nystatin on the cellular uptake of the different A2 peptide formulations.

Cells incubated with fA2 revealed high variance in the fluorescent intensities (Fig. 5A). At 4 °C CLSM studies showed little active uptake and only the evenly distributed cellular fluorescence contributed to the signal. The FACS results showed cells with highly diverse fluorescence intensity. Hence, the mean fluorescence intensities observed in cells show high standard deviations.

b.End3 and BAEC cells incubated at 37 °C with liposomes fluorescently labeled with rhodamine-DPPE and targeted with P2A2 revealed high fluorescent intensities (Fig. 5B). Cytochalasin D

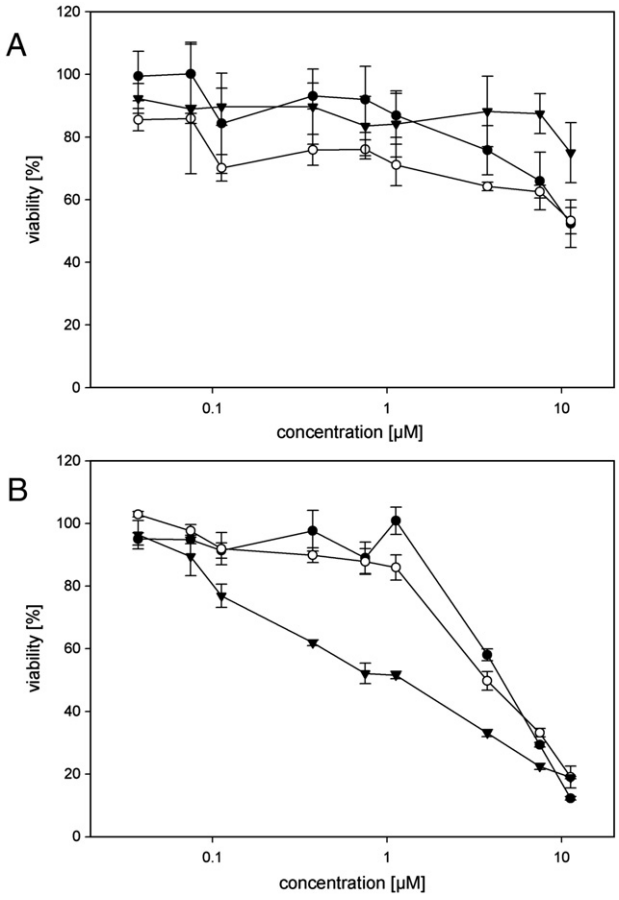

Fig. 3. Viability of (A) mouse brain capillary endothelial cells (b.End3) and (B) bovine aortic endothelial cells (BAEC) exposed to increasing concentrations of the peptides fA2 (●), P2fA2(○), and of P2fA2-liposome complexes (▼) with a $C_{l/p}$ of 1000.

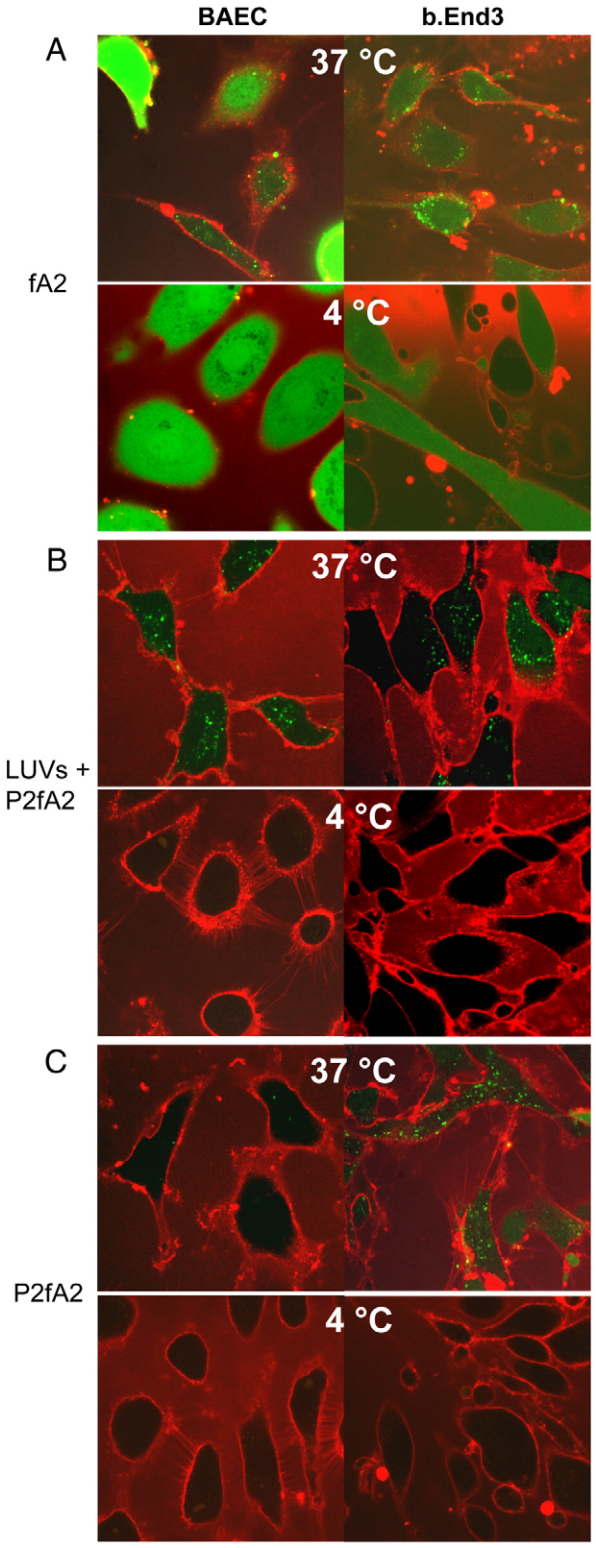

Fig. 4. CLSM images of mouse brain capillary endothelial cells (b.End3, right) and bovine aortic endothelial cells (BAEC, left) exposed to (A) 2 μM fA2, (B) 1 μM P2fA2-POPC liposome complexes ($C_{l/p}$ of 1000), or (C) 1 μM P2fA2 at 37 °C or 4 °C. One drop of 0.5% trypanblue in PBS was carefully put onto the surface of the microscopic sample. The red fluorescence of trypanblue on the cell membrane documents the viability of the cells. Pictures were taken after incubation for 1 h.

efficiently reduced the signal to the level of cells incubated at 4 °C, while chlorpromazine and nystatin had no effect. These observations point to non-clathrin, non-caveolin-mediated endocytosis as major uptake route for peptide-tagged liposomes.

Fig. 5C confirms that P2fA2 micelles were efficiently internalized into b.End3 cells when incubated at 37 °C. Cytochalasin D and chlorpromazine efficiently blocked this uptake, while nystatin had no effect. In contrast, the uptake of micelles into BAEC cells was very low and modification of the incubation conditions had little influence. These findings are consistent with results of CLSM studies performed under the same conditions (data not shown). The results imply, that micellar P2fA2 enters b.End3 cells via a clathrin-mediated endocytotic uptake route, which is not active or at least strongly reduced in BAEC

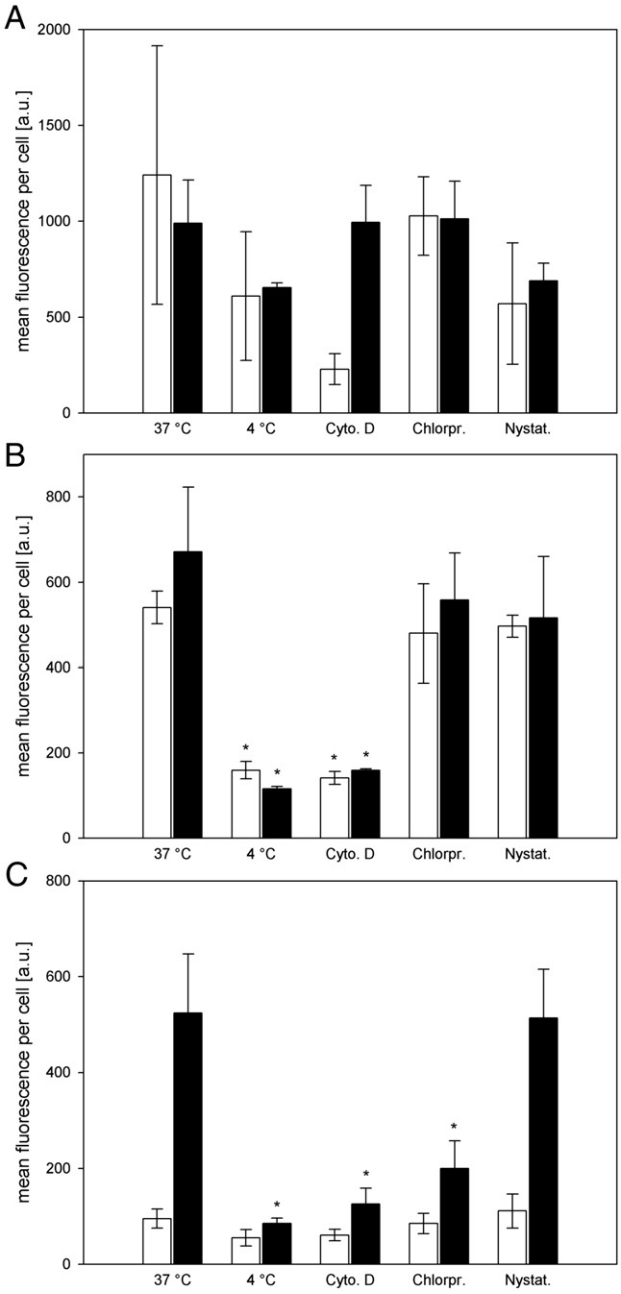

Fig. 5. FACS analysis of mouse brain capillary endothelial cells (b.End3, black) and bovine aortic endothelial cells (BAEC, white) exposed for 1 h to (A) 2 μM fA2 (FL1, 500 V detector voltage), (B) 1 μM P2A2 and 0.1% Rhodamine-DPPE POPC liposomes ($C_{l/p}$ of 1000, FL2, 550 V detector voltage), or (C) 1 μM P2fA2 (FL1, 550 V detector voltage) at 37 °C, 4 °C, in presence of 2 mg/ml cytochalasin D, 10 mg/ml chlorpromazine, or 25 mg/ml nystatin. Cells for analysis were gated by scatter plots and a minimum of 10,000 events per sample was analyzed. Each experiment was performed at least twice in duplicates or triplicates. Means ± standard deviation are indicated. Values significantly ($p<0.05$) different from control (internalization at 37 °C) as determined by Student's *t*-test are marked with asterisk.

cells. As this may be related to a general deficiency in clathrin-mediated endocytosis or the absence of specific uptake mediating structures for micellar P2fA2, we examined the uptake of transferrin into BAEC cells as an example for clathrin-mediated endocytosis [44].

3.5. Uptake of transferrin (FACS)

FACS studies showed efficient internalization of transferrin into b.End3 cells at 37 °C, which is inhibited at 4 °C and in the presence of cytochalasin D and significantly reduced by chlorpromazine (Fig. 6). This confirms the existence of clathrin-mediated endocytosis in b.End3 cells. The uptake of transferrin in BAEC at 37 °C cells is reduced compared to b.End3 cells. At 4 °C and the presence of cytochalasin D the signal is further decreased but the effect of chlorpromazine was not significant. This is indicative of an endocytotic mechanism, that is not dependent on clathrin.

4. Discussion

Although the application of micelles and liposomes to overcome cellular boundaries is highly favorable, the specificity and uptake of the high molecular weight carriers is poor in most cells. As shown in this study, the membrane-translocating sequence A2 mediates the cellular uptake of both particles. The uptake is unspecific for liposomes, but cell-selective for peptide micelles and mediated by different endocytotic mechanisms.

Monomeric fA2 is readily taken up into endothelial cells of brain capillaries (b.End3) as well as of large blood vessels (BAEC) by at least two functionally different mechanisms. The vesicular fluorescence is blocked at low temperature pointing to an endocytotic mechanism, while the cytosolic fluorescence observed even at low temperature proves the existence of a second non-active internalization mechanism. As an escape of fA2 from the vesicles can be ruled out and the integrity of the plasma membrane is conserved as assayed by trypanblue exclusion, the cytosolic fluorescence is no artefact as observed in earlier CPP studies, where the diffuse fluorescence pattern was caused by redistribution upon cell fixation [45]. Similar observations have been made with the TAT peptide and mouse myoblasts [18] and with penetratin, nona-arginine (R9) and TAT on HeLa cells [5]. The underlying processes of the rapid internalization referred to as transduction are still in question. However, formation of membrane-soluble ion pair complexes with negatively charged membrane constituents and a subsequent internalization driven by the transmembrane potential may play a role [11,46,47].

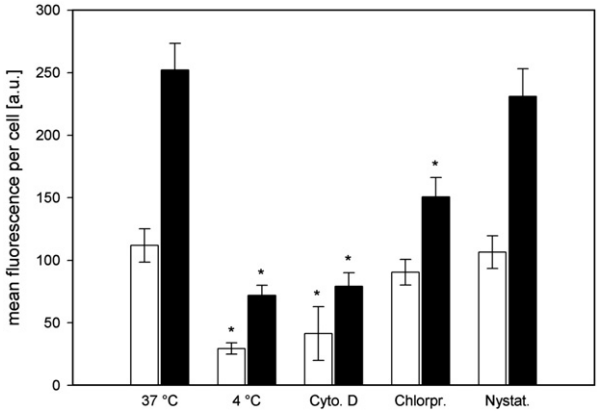

Fig. 6. FACS analysis of mouse brain capillary endothelial cells (b.End3, black) and bovine aortic endothelial cells (BAEC, white) exposed for 1 h to 250 nM transferrin (FL1, 550 V detector voltage) at 37 °C, 4 °C, in presence of 2 mg/ml cytochalasin D, 10 mg/ml chlorpromazine, or 25 mg/ml nystatin. Cells for analysis were gated by scatter plots and a minimum of 10,000 events per sample was analyzed. Each experiment was performed at least twice in duplicates or triplicates. Means±standard deviation are indicated. Values significantly ($p<0.05$) different from the control (internalization at 37 °C) as determined by Student's *t*-test are marked with asterisk.

Incubation of b.End3 and BAEC cells with P2fA2-tagged liposomes resulted exclusively in strong vesicular fluorescence, which is blocked at low temperature. This clearly points to an endocytotic uptake mechanism, which is not cell selective. A shift of the internalization mechanism to endocytosis has also been observed for TAT and penetratin upon coupling to proteins or liposomes [18,48]. In contrast to fA2 and peptide liposome complexes, micellar P2fA2 showed distinct cell selectivity. P2fA2 micelles entered b.End3 cells in an endocytotic manner, whereas the uptake into BAEC cells was found to be low.

The striking difference in the extent of micellar and liposomal internalization prompted us to examine the underlying endocytotic mechanisms. P2fA2-tagged liposomes were found to be internalized by an endocytotic process that was independent of clathrin and caveolin in both cell lines. In difference to liposomes, P2fA2 micelles are internalized into b.End3 cells via clathrin-dependent endocytosis as the internalization was significantly decreased by cytochalasin D and chlorpromazine. A mono-stearylated octa-arginine has been found to enter NIH/3T3-cells in the same way [49]. Though the structure was not examined in detail, one might expect that the lipidated oligo-arginine organizes in a similar micellar structure as P2fA2.

The intriguing mechanistic switch observed between the monomeric and particulate peptides may be related either to the size difference between micelles and liposomes and/or the different peptide densities on the surface. Micellar P2fA2 is characterized by a peptide-surface density of 5.38×10^{-4} P2A2/Å^2,while liposomes tagged with P2fA2 have a more than 10 times lower peptide-surface density of 3.18×10^{-5} P2A2/Å^2.

The different characteristics may lead to selective interactions with cell surface HSPGs and subsequent activation of different uptake mechanisms. Ligand-induced clustering of syndecans, an HSPG class with type I transmembrane core proteins, enhances the rate of endocytosis [50]. An increase in endocytotic activity is suggested upon presentation of multivalent ligands [51]. P2A2 tagged particles might act in the same way. While the internalization of lipoproteins via the LDLr in a clathrin-dependent manner is well known, syndecans have been shown to mediate the clathrin-independent endocytosis of lipoproteins and apoE-VLDL bound to lipoprotein lipase [50].

Whereas the internalization of A2 tagged sterical stabilized liposomes into human fibroblasts is LDLr-independent [16], it is conceivable that the high local concentration of ligands as seen in P2fA2 micelles may increase the affinity for the LDLr because of avidity effects. It has been shown, that the high affinity of apoE-containing lipoproteins to the LDLr results from the potential to form multiple interactions with the receptor [52]. However, the cellular structures which mediate binding and trigger the subsequent internalization of micellar P2fA2 remain to be elucidated.

The influence of the endocytosis inhibitors upon the P2fA2 uptake into BAEC is low and little differentiated, which can be related to the generally low uptake of P2fA2 micelles. To test the existence of clathrin-mediated endocytosis in these cells, we compared the uptake of transferrin in both cell lines. The internalization of transferrin is widely accepted as a classical example for clathrin-mediated endocytosis [44]. FACS studies show efficient internalization of transferrin into b.End3 cells, which is inhibited in the presence of cytochalasin D and significantly reduced by chlorpromazine. This is not only a proof for the activity of the used inhibitors but also confirms the existence of clathrin-mediated endocytosis. The uptake of transferrin in BAEC cells was reduced compared to b.End3 cells and the effect of chlorpromazine was not significant. This denotes that the observed preferance of P2A2 micelles for b.End3 cells may be attributed to a general deficiency of clathrin-mediated endocytosis in BAEC cells.

No involvement of caveolae in the internalization of monomeric fA2, P2fA2 micelles or liposomes tagged with P2A2 was observed

although the expression of caveolin was shown for b.End3 cells [36] as well as for BAEC cells [53] and a caveolin-mediated uptake of TAT fusion proteins in HeLa cells has been reported [54,55].

In summary, our results show that not only the uptake mediating compound, but also the structure of the carrier mediates target specificity and affects the route of cellular uptake. Free monomeric fA2 is taken up by at least two functionally different mechanisms. Besides an endocytotic route, a second non-active pathway exists, which leads to a rapid distribution of fA2 into the cytoplasm. The uptake of carriers tagged with A2 is mediated by endocytotic processes. However, the involved cellular structures are different. While micellar P2fA2 is internalized selectively by capillary endothelial cells via clathrin-mediated endocytosis, the uptake of liposomes tagged with P2fA2 seems to be not cell-specific and is clathrin- and caveolin-independent. The efficient internalization of micellar P2fA2 into capillary endothelial cells but low uptake into endothelial cells of large vessels may provide a basis for the development of more site-specific carrier systems. However, endosomes formed after clathrin-mediated endocytosis eventually fuse with lysosomes where degradation of the internalized material occurs [56]. Hence, the advantage of site specificity is likely opposed by a rather unfavorable intracellular fate of the peptide micelles. The way in which the size of the particle and the peptide surface density influence the recognition of cell surface constituents and determine the uptake route remains to be examined in detail.

Acknowledgments

This work was supported by the Deutsche Forschungsgemeinschaft (grant FG 463, DA 324/5-1,5-2).

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