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Research Article

Low-density lipoprotein receptor-mediated endocytosis of PEGylated nanoparticles in rat brain endothelial cells

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Abstract. Poly(methoxypolyethyleneglycol cyanoacrylate-co-hexadecylcyanoacrylate) (PEG-PHDCA) nanoparticles have demonstrated their capacity to diffuse through the blood-brain barrier after intravenous administration. However, the mechanism of transport of these nanoparticles into brain has not yet been clearly elucidated. The development of a model of rat brain endothelial cells (RBEC) in culture has allowed investigations into this mechanism. A study of the intracellular trafficking of nanoparticles by cell fractionation and confocal microscopy showed that nanoparticles are internalized by the endocytic pathway. Inhibition of the caveolae-mediated pathway by preincubation with filipin and nystatin did not modify the cellular uptake of the nanoparticles. In contrast, chlorpromazine and NaN_3 pretreatment, which interferes with clathrin and energy-dependent endocytosis, caused a significant decrease of nanoparticle internalization. Furthermore, cellular uptake experiments with nanoparticles preincubated with apolipoprotein E and blocking of low-density lipoprotein receptors (LDLR) clearly suggested that the LDLR-mediated pathway was involved in the endocytosis of PEG-PHDCA nanoparticles by RBEC.

Keywords: Rat brain endothelial cells, PEGylated nanoparticles, poly(alkylcyanoacrylate), clathrin-coated pits, low-density lipoprotein receptor-mediated endocytosis, apolipoprotein E.

Introduction

The brain is very efficiently protected by the blood-brain barrier (BBB), consisting of endothelial cells adjoined with continuous tight junctions. This continuous physical barrier and a low activity of vesicular transport and pinocytosis combined to give the BBB very low permeability. In fact only unionized, lipophilic and small-sized molecules can diffuse through the BBB. A few essential compounds such as hexoses, amino acids, neuropeptides and proteins can freely pass through endothelial cell membrane by means of specific transport systems [1]. Hence, this barrier

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restricts the transcellular transport of free drugs, thus hindering the pharmacological effect for numerous brain disorders treatments. To improve the bioavailability of drugs active in the brain, physical treatment comprising osmotic or chemical opening of BBB or direct intracerebroventricular drug injection leads to a nonspecific permeability and is therefore highly toxic for the patients, although drug transport across the BBB is temporarily increased [2, 3]. The chemical modification of drugs by the attachment of hydrophobic group or by the conjugation of the drug with a transporter specific to the BBB has been used as a non-invasive strategy [4]. However, this approach often involves cleavable linkers and an appropriate 1:1 stoichiometry of carrier to drugs, which results in the decrease of therapeutic efficiency and/or insufficient carrying capacity [5]. Invariably, a linker strategy that works for a given drug-carrier complex will not work for another drug and, in this case, an alternative linker strategy must be devised [5]. Therefore, the incorporation of the non-transportable drug into colloidal carriers, such as liposomes or nanoparticles, has been considered to extend the carrying capacity in terms of the number of drug molecules per carrier and to have more flexible and drug-independent nanotransporters.

Recently, *in vivo* studies have shown that PEGylated nanoparticles composed of an amphiphilic copolymer consisting of poly(ethylene glycol) (PEG) as hydrophilic chains and a poly(hexadecylcyanoacrylate) (PHDCA)-hydrophobic block, were able to penetrate into both healthy rat brain and brain glioma [6–9]. After nanoprecipitation of this poly(methoxypolyethyleneglycol cyanoacrylate-co-hexadecylcyanoacrylate) (PEG-PHDCA) copolymer, PHDCA formed the particle core, while PEG provided a protective cloud at their surface [10]. As a result, PEGylated nanoparticles exhibited a long circulation time in blood due to reduced opsonization [11]. Interestingly, PEG-PHDCA nanoparticles were found to accumulate in brain, both by diffusion/convection because of their prolonged blood circulation time, and also due to the specific affinity of the surface of these nanoparticles for the endothelial cell membrane of the BBB [9]. To demonstrate the translocation of colloidal systems across the BBB, a relevant rat BBB *in vitro* model has been established in transwells with primary cultures of rat brain endothelial cells (RBEC) and astrocytes [8]. This *in vitro* rat BBB model retained the specific brain endothelial cell protein, P-glycoprotein and tight junction proteins such as occludin and ZO-1. Moreover, the model demonstrated high values of transendothelial electrical resistance and low permeability coefficients of marker molecules. PEG-PHDCA nanoparticle transport was also confirmed in this model. The passage of PEG-PHDCA nanoparticles through this *in vitro* model was higher than that of PHDCA nanoparticles, and increased with time, thus showing a good *in vitro*/*in vivo* correlation [12]. Experiments on cell internalization and intracellular distribution of PEG-PHDCA nanoparticles in RBEC suggested that cellular uptake of nanoparticles took place by specific endocytosis [13]. Even in the brain, endocytosis is essential for the turnover of proteins and lipids of the plasma membrane, transmission of extracellular signals and uptake of many essential nutrients. The recent literature shows that cellular uptake is not restricted to a single type of endocytosis but that different mechanisms have been demonstrated: clathrin-dependent endocytosis [14] and clathrin-independent endocytosis (raft caveolae-dependent endocytosis) [15].

Polysorbate 80 (PS 80)-coated polybutylcyanoacrylate (PBCA) nanoparticles have been used as a carrier to enhance brain penetration of drugs such as dalargin [16, 17], loperamide [18], tubocurarine [19], the NMDA receptor antagonist MRZ2/576 [20] and doxorubicin [21]. One hypothesis to explain the uptake of nanoparticles is that endocytosis by endothelial cells of the BBB occurs via the low-density lipoprotein receptor (LDLR) gene family: it is assumed that 'Trojan Horse' lipoprotein-like particles are formed as a consequence of the adsorption of apolipoprotein E (ApoE) and B (ApoB) onto the surface of the nanoparticles [22–24]. Another pathway that could be implicated in brain delivery is caveolae, abundant vesicular transporters in endothelial cells, which comprise smooth invaginations of the plasma membrane [25]. These caveolae could be involved in the LDL transcytosis pathway across BBB as a non-classical pathway of LDLR action [26]. Indeed, it has been reported that brain-targeted cationized albumin (CBSA)-coupled liposomes undergo caveolae-mediated endocytosis as evidenced by the reduction of cellular uptake after incubation with filipin, an inhibitor of caveolae, in porcine brain capillary endothelial cells [27]. However, the transport mechanism of colloidal carriers into brain remains controversial: it is unclear whether they penetrate by LDLR-mediated endocytosis, caveolae-mediated pathway or nonspecific tight junction opening due to a toxic effect.

To understand the cellular uptake of PEG-PHDCA nanoparticles by RBEC more clearly, we investigated here the overall mechanism by cell fractionation after different time intervals and also by specific inhibition studies using inhibitors such as filipin and nystatin to disrupt the caveolae-mediated pathway, and chlorpromazine and sodium azide to interfere with clathrin-coated pit assembly implicated in LDLR gene

family and energy-dependent endocytosis, respectively. Cellular uptake experiments with ApoE-preincubated PEG-PHDCA nanoparticles and LDLR blocking experiment using an LDLR-specific monoclonal antibody were performed to demonstrate the LDLR-mediated endocytosis of the nanoparticles into RBEC.

Since the BBB consists primarily of endothelial cells forming the walls of brain microvessels and since endothelial cells specifically regulate and largely contribute to the transport of substances into the brain [5], these cells may be considered as responsible for the ability of certain drug carriers to cross the barrier. Thus, in the present study, all the experiments were performed in cultured microvessel brain endothelial cells of rat as used earlier in the *in vitro* model of BBB published in this journal [12].

Materials and methods

Materials. PEG-PHDCA 1:4 copolymer was prepared by the procedure described previously [6, 10]. PEG-PHDCA 1:4 were synthesized by condensation of methoxypoly(ethylene glycol) cyanoacetate (MePEG, molecular weight 2000) with *n*-hexadecyl cyanoacetate (HDCA) in ethanol, in presence of formalin and pyrrolidine [9, 10]. Radiolabeled [^{14}C]PEG-PHDCA 1:4 copolymer was synthesized at the Commissariat à l'Energie Atomique (Saclay, France). Its specific activity was 2.8 μCi/mg. All chemicals were of analytical grade.

Biodegradable cyanoacrylate nanoparticle preparation and characterization. Nanoparticles were obtained by the nanoprecipitation method described previously [6]. To obtain fluorescent nanoparticles, 50 μl nile red (0.1 g/l; Molecular Probes, Oregon, USA) was added to the solution of copolymer (10 mg) in acetone (0.95 ml) and this solution was mixed with 2 ml of an aqueous solution containing 0.5% (w/v) Pluronic F68 (Fluka, France). Nanoparticles were formed immediately. After evaporation of the acetone, nanoparticles were purified by centrifugation (150 000 *g*, 1 h, 4°C, Beckman Coulter, Inc., CA, USA). The pellet was resuspended in an appropriate volume of water. The same procedure was used with the [^{14}C]PEG-PHDCA copolymer to obtain radiolabeled nanoparticles. The mean diameters were 140±42 nm and 146±41 nm and zeta potential were −20±2 and −20±1 mV for [^{14}C]PEG-PHDCA and nile red fluorescent PEG-PHDCA nanoparticles, respectively. These data were obtained by quasi-elastic light scattering, at 90° (Coulter N4MD, Beckman Coulter) and by Zeta sizer (Zeta sizer 4, 7032 Multi 8 correlator; Malvern Instruments, Orsay, France).

Rat brain endothelial cell culture. The RBEC were obtained by the primary culture method as described previously [12] with slight modification. Briefly, the 2-week-old Sprague-Dawley rat brain cortex was used. After removing the cerebellum and white matter, the meninges and choroids plexus were peeled off and the gray matter was chopped. The first enzyme digestion was carried out in the collagenase type II (270 U/ml, Worthington Biochemical corp., NJ, USA) solution supplemented with 20 U/ml DNase I (deoxyribonuclease I, Sigma-Aldrich) and 3.64 μg/ml TLCK (tosyl-lysine-chloromethyl-ketone, Sigma-Aldrich) for 2 h at 37°C with gentle shaking. The digested suspension was centrifuged at 1000 *g*, for 5 min at 4°C and the pellet was resuspended with 25% BSA (Sigma-Aldrich) followed by density-dependent centrifugation at 1500 *g*, for 15 min at 4°C. This step separated the heavier capillary fragments from the myelin, astrocytes, neurons and other single cells. The pellet was resuspended for the second enzyme digestion in collagenase/dispase (0.1% neutral protease dispase II, Roche Diagnostics, Switzerland) supplemented with 20 U/ml DNase I and 3.64 μg/ml TLCK and was incubated for 1.5 h at 37°C with occasional shaking. The suspension resulting from the second enzyme digestion was filtered through a 10-μm pore size nylon mesh and the capillary fragments on the mesh were washed three times with PBS. Finally, the capillaries were seeded into a 60-mm petri dish (Corning Costar, MA, USA) coated with collagen type IV (100 μg/ml, Sigma-Aldrich) and were grown in a humidified atmosphere with 5% CO_2 at 37°C. The culture medium was the EBM-2MV (Cambrex, Belgium) basal medium containing 10% fetal bovine serum (FBS) and antibiotics (gentamycin/amphotericin) supplemented with 3 μg/ml puromycin (Sigma-Aldrich) for 3 days [28]. From the 4th day, the culture medium was changed to the EBM2-MV Bullekit (Cambrex, Belgium) and renewed every other day. Immunocytochemical staining with anti-von Willebrand factor (DAKO Cytomation, Trappes, France), a specific marker of endothelial cells, after 1 week from the seeding of the capillaries in confluence, showed high purity and characteristics of endothelial cells in culture (data not shown). The staining protocol was carried out according to the DAKO LSAB2 kit.

For experiments, RBEC at first passage were seeded onto collagen type IV-coated six-well plates (Corning Costar). The culture medium, EBM-2MV Bullekit (Cambrex, Belgium) was changed routinely until the cells reached confluence.

Uptake experiments. To measure cellular uptake kinetics, 20 μg/ml of [^{14}C]PEG-PHDCA 1:4 nanoparticles suspensions were prepared in the transport medium (TM, 5% FBS in EBM-2MV). The cells were washed with 2 ml PBS and the nanoparticles were then added for different incubation times (5, 10, 20, 40, 60 or 120 min) at 37°C in a humidified atmosphere with 5% CO_2.

To study the dose-dependence of uptake of ApoE-[^{14}C]PEG-PHDCA nanoparticles, [^{14}C]PEG-PHDCA nanoparticles were preincubated with ApoE according to a protocol adapted from the literature [23]. ApoE (Calbiochem, Darmstadt, Germany) solutions at different concentrations (0.05, 0.3, 1, 3, and 10 nM) were incubated with 20 μg/ml of the nanoparticles in PBS for 1 h in the presence of protease inhibitors cocktail (Sigma-Aldrich) under gentle stirring at room temperature. This nanoparticle suspension was then incubated with RBEC for 20 min at 37°C in a humidified atmosphere with 5% CO_2.

The incubation was stopped by removing the nanoparticles, followed by washing the cells three times with 2 ml cold PBS (4°C). The cells were lysed by adding a 0.2 M NaOH solution containing 1% SDS and the radioactivity was counted (Beckman model LS 6000TA). Uptake was expressed as the amount (μg) of cell-associated nanoparticles per unit weight (mg) of cell proteins. The experiments were performed in triplicate and the results are the mean ± SD.

Cell fractionation studies. The cell fractionation was performed as described previously [13] with slight modification. Briefly, after different incubation times (5, 10, 20, 40, 60 and 120 min) of radiolabeled nanoparticles with RBEC (in a humidified atmosphere; 5% CO_2), cells were first treated by 0.1% protease (Sigma-Aldrich) in protective buffer (0.25 M sucrose, 20 mM HEPES, 2 mM potassium phosphate, 0.24 mM EGTA and 10 mM $MgCl_2$) for 15 min at 4°C to separate the nanoparticles adsorbed onto the cell membrane [29]. Secondly, the cell membrane was permeabilized using digitonin (0.003% in PBS for 15 min at 4°C) to extract the cytoplasmic fraction. Thirdly, cells were treated by 1% Triton X-100 for 20 min at 4°C to permeabilize intracellular endosomal/lysosomal vesicles. Finally, the remaining Triton-insoluble fraction corresponded to the nuclear fraction. After each step, centrifugation was carried out to separate the soluble fraction from the cell suspension and the pellet was washed with PBS. The radioactivity of each fraction (*i.e.*, membrane, cytosol, endosomal/lysosomal vesicles and nucleus) was counted (Beckman model LS 6000 TA). All experiments were performed in triplicate and the results are the mean ± SD.

To estimate the effectiveness of this methodology to recover these intracellular fractions, lactate dehydrogenase (LDH kit, Roche diagnostic, Switzerland) and cathepsin B (Cath. B) were used as markers of the cytosol and vesicular compartments, respectively.

At the 0.003% (w/v) of digitonin, the percentage of total cellular LDH release was 76±1% and Cath. B activity was 15±4% in the purified cytosol fraction. The vesicular fraction permeated by 1% Triton X-100 represented 83±8% of Cath. B activity and 14±2% of LDH release of each total enzyme. Thus, treatment by 0.003% digitonin allowed the majority of the cytosol content to be recovered with minimal damage to the intracellular vesicles and limited contamination of the other fractions, as previously reported [13].

Western blotting. RBEC were scraped from the six-well plates and solubilized with lysis buffer [10 mM Tris, pH 7.5, 5 mM EDTA, 125 mM NaCl, 1% Triton, 0.1% SDS and protease inhibitors, 10 μg/ml aprotinin (Sigma-Aldrich) and 10 μg/ml leupeptin (Sigma-Aldrich)]. To determine the protein concentration of the cell lysate, the colorimetric Bicinchoninic Assay Kit (Uptima, Interchim, Montlucon, France) was used. Proteins (10 μg) were migrated on 8% or 12% SDS-polyacrylamide gel and electrophoretically transferred to a nitrocellulose membrane. For the LRP-1 blot, the procedure was performed in nonreducing conditions. Blots were blocked with 10% (w/v) skimmed milk in Tris buffer saline. They were rinsed and incubated with primary antibodies, anti-goat LDLR antibody (Santa Cruz Biotechnology, CA, USA) at 1:200 dilution, anti-mouse LDLR-related protein-1 (anti-LRP-1) light chain antibody (Calbiochem, Darmstadt, Germany) with 1 μg/ml or anti-rabbit caveolin-1 antibody (Sigma-Aldrich) at 1:2000 dilution, for 3 h at room temperature, followed by a peroxidase-conjugated anti-goat, mouse or rabbit IgG (DAKO Cytomation) as secondary antibody. The immunoreactive bands were visualized by an enhanced chemiluminescent system (Amersham Bioscience, Saclay, France).

Inhibition studies. Brain capillaries were seeded on a 10-mm-diameter plastic cover slip coated with collagen type IV (100 μg/ml) and then incubated for 1 week to allow the RBEC to reach confluence. To determine uptake of nanoparticles, the cells were incubated with nile red fluorescent nanoparticles (20 μg/ml) for 20 min at 37°C in a humidified atmosphere with 5% CO_2. Endocytosis inhibitors: 3 μg/ml filipin (Sigma-Aldrich), 3 μg/ml nystatin (Sigma-Aldrich), 30 μM chlorpromazine (Sigma-Aldrich) and 0.1% NaN_3, were prepared in the transport medium (TM, EBM-2MV supplemented with 5% FBS) and prewarmed at 37°C. Each inhibitor solution was preincubated for 10 min at 37°C with the cells. The nile red fluorescent nanoparticle suspension (final concentration 20 μg/ml) was added to the treated cell culture and incubated at 37°C for 20 min in 5% CO_2. At the end of the incubation time, the nanoparticle suspension was removed and the RBEC monolayer was washed three times with PBS and fixed with 1% paraformaldehyde for 15 min at room temperature. Cells were examined under a laser scanning confocal microscope (Zeiss LSM-510 META) equipped with a 1-mW helium/neon laser and a Plan Aprochromat ×20 objective with a numerical aperture of 0.75 at the median z step between the top and the bottom of the monolayer to ensure that intracellular nanoparticles only were observed, with an optical section thickness of 2.5 μm. The pinhole size was set at 1.0 Airy unit (67 μm diameter). Red fluorescence was collected with the META detector (563–660 nm) under 543-nm laser illumination.

LDLR blocking studies. The anti-LDLR monoclonal antibody (mAb) (Calbiochem) was used to block the ligand-binding domain, extracellular region of the N terminus, which comprises the cysteine-rich repeat of LDLR [30]. Capillaries were seeded onto cover slips coated with collagen type IV (100 μg/ml) and the experiment was performed with confluent RBEC 1 week after seeding. Cells were incubated with or without 2.5 μg/ml anti-LDLR mAb for 1 h at 4°C [31, 32], after which the nile red fluorescent PEG-PHDCA nanoparticles were added (20 μg/ml) for 20 min at 37°C in 5% CO_2. The cells were washed three times with PBS and fixed with 1% paraformaldehyde for 15 min. For fluorescence analysis, the LSM 510 Zeiss confocal inverted microscope was used. For quantification of fluorescence images, three independent experiments were analyzed by the Image J program. Confocal microscopic images were directly saved in the LSM manufacturer's format and exported to TIF file. The image color was made RGB Split and the type was adjusted 8 bit (256 gray values). The background color in different images was selected with a threshold to normalize all images for the luminosity of the red color channel. Mean gray value (MGV) in set measurement was analyzed from the remaining pixels. The obtained MGV was divided by cell number in the chosen image and this fluorescence intensity per cell was compared.

Results

Intracellular distribution as a function of time. To study the translocation mechanism of PEG-PHDCA nanoparticles through RBEC, the intracellular distribution of radiolabeled nanoparticles within RBEC was studied as a function of time. Cellular uptake kinetics of [^{14}C]PEG-PHDCA nanoparticles with RBEC showed a saturable binding at 2.54±0.08 μg/mg protein after 60-min incubation (Fig. 1a). To investigate intracellular distribution of the nanoparticles, we performed the cell fractionation after different periods of nanoparticle incubation with RBEC. After 5 min 76.6±5.12% of [^{14}C]PEG-PHDCA nanoparticles were in the membrane fraction. This proportion decreased steadily down to 50.8±4.5% after 120 min. In contrast, 12.0±3.5% of the cell-associated nanoparticles were in the vesicular fraction at 5 min but this proportion increased remarkably up to 37.6±4.6% at 120 min without significant change in the cytoplasm fraction and in the 1% Triton X-100-insoluble fraction, which remained within the range of 7.3±0.5 to 10.6±0.4% and of 2.2±1.0 to 4.4±0.4%, respectively (Fig. 1b).

Expression of transporters for endocytosis in the membrane of RBEC. The expression of two well-known members of the LDLR gene family, *i.e.*, LDLR and LRP-1 in RBEC was revealed by Western blotting. LDLR showed a specific band at 120 kDa and LRP-1 a light chain at 85 kDa. Caveolin-1, a major structural protein of caveolae was also detected at 24 kDa (Fig. 2). In addition, a very abundant expression of caveolin-1 indicated that it is a ubiquitous transport protein in RBEC.

Inhibition of clathrin and caveolae-mediated pathways. We investigated the cellular uptake of the nile red fluorescently labeled PEG-PHDCA nanoparticles by confocal microscopy studies after 20 min of incubation (Fig. 3a). The fluorescent nanoparticles appeared as perinuclear punctate spots in the cytoplasm. Thus, confocal microscopy experiments with fluorescent nanoparticles correlated well with the preceding data on the intracellular distribution obtained using radiolabeled nanoparticles. To elucidate whether the uptake of PEG-PHDCA nanoparticles

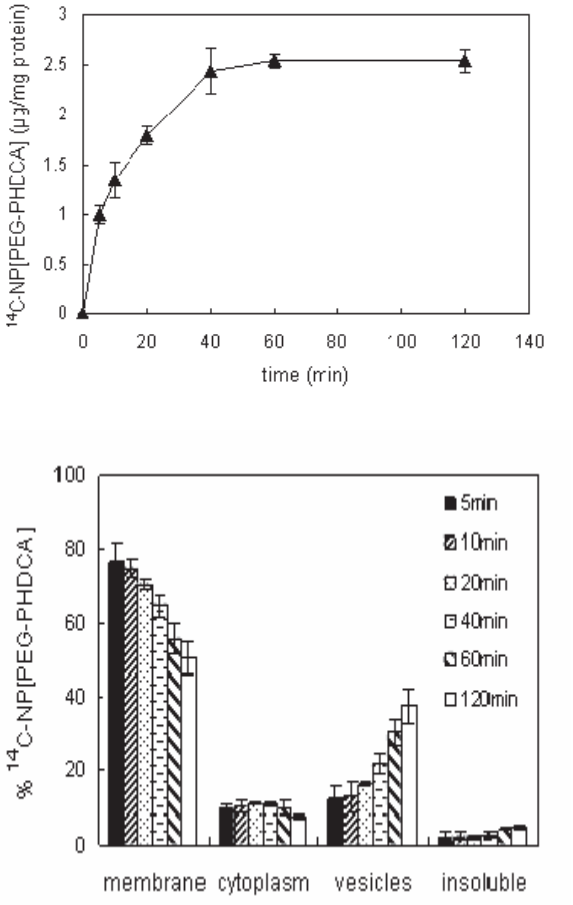

Figure 1. Cell uptake (a) and intracellular distribution (b) over time of [^{14}C]poly(methoxypolyethyleneglycol cyanoacrylate-co-hexadecylcyanoacrylate) (PEG-PHDCA) nanoparticles in rat brain endothelial cells (RBEC). The data from cell fractionation are represented by percentage of radioactivity of nanoparticles in each fraction. The values are means ± SD in triplicate.

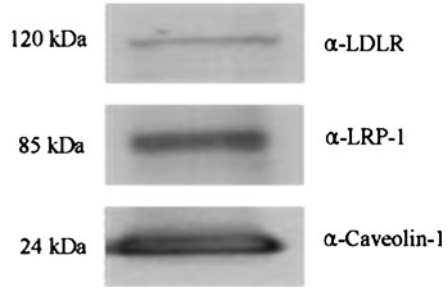

Figure 2. Detection of low-density lipoprotein receptor (LDLR), LDLR-related protein-1 (LRP-1) and caveolin-1 in RBEC by Western blotting using specific antibodies as described in the Materials and Methods section.

involves on the caveolae or clathrin, inhibition studies were performed with filipin and nystatin, which are sterol-binding agents known to interfere with the caveolae-dependent pathway [33]. Moreover, chlorpromazine was used to disrupt the assembly of clathrin-coated pits at the cell membrane [34], and NaN_3 to inhibit the energy-dependent receptor-mediated endocytosis. The data obtained showed that a 10-min pretreatment with either 3 μg/ml filipin or nystatin at 37°C, did not significantly affect the uptake of fluorescent PEG-PHDCA nanoparticles by RBEC (Fig. 3b and c). However, as a result of a pretreatment by 30 μM chlorpromazine, the uptake of the nanoparticles was substantially reduced (Fig. 3d). In addition, NaN_3-pretreated cells showed a dramatic decrease in the nanoparticle fluorescence signal (Fig. 3e).

Studies of ApoE- and LDLR-mediated endocytosis. To investigate the receptor-mediated pathway of nanoparticle translocation, ApoE, one of the ligands recognized by the LDLR gene family, was preincubated with PEG-PHDCA nanoparticles at different concentrations before incubation with RBEC. Cellular uptake of ApoE-preincubated [^{14}C]PEG-PHDCA nanoparticles increased with ApoE concentration up to 3.1±0.2 μg/mg protein at 1 nM ApoE and thereafter remained at a plateau value (Fig. 4). To confirm that LDLR was involved in nanoparticle endocytosis, confocal microscopy experiments were performed with PEG-PHDCA nanoparticles fluorescently labeled with nile red in the presence or absence of an anti-LDLR mAb. Figure 5 shows that the cell-associated fluorescence was dramatically reduced when the LDLR was blocked (1 h incubation of 2.5 μg/ml anti-LDLR mAb). Quantitative analysis demonstrated that the fluorescence intensity decreased sixfold from 43.4±5.6 to 7.4±2.7 pixels after incubation with anti-LDLR mAb (Fig. 5c).

Discussion

In the reproducible primary RBEC culture, the uptake of PEG-PHDCA nanoparticles by the cells was saturable with time, suggesting an active transport (Fig. 1a). The study of the intracellular distribution as a function of time demonstrated that the mechanism of cell internalization of PEG-PHDCA nanoparticles was clearly endocytosis because the proportion of cell-associated radioactivity in the vesicular fraction increased in a time-dependent manner without any significant change in the radioactivity content of the cytoplasm or the 1% Triton-insoluble fraction over the same time interval (Fig. 1b). Furthermore, the confocal microscopy images of fluorescently labeled PEG-PHDCA nanoparticles in cells showed that the fluorescence was concentrated in a punctuate pattern around the nucleus, suggesting an intracellular endo/lysosomal localization (Fig. 3a).

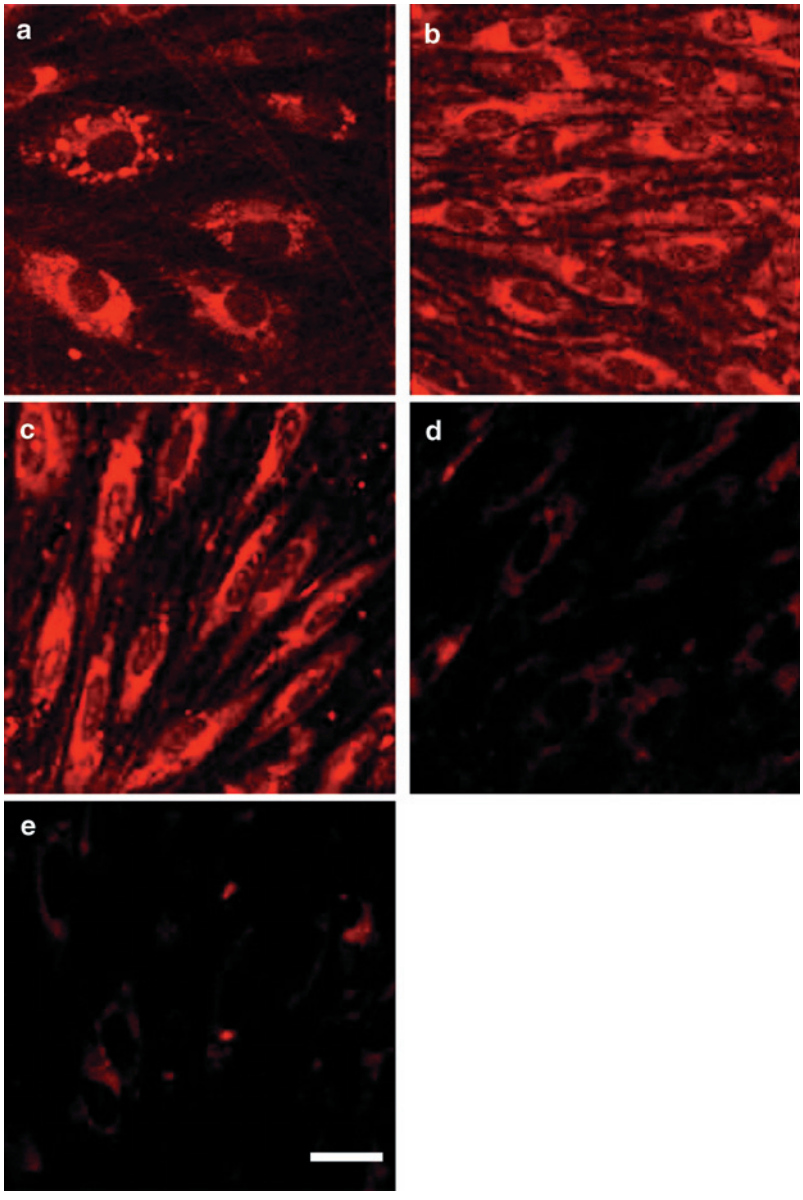

Figure 3. Confocal microscopy observations of RBEC after incubation (20 min; 37°C) with nile red fluorescently labeled PEG-PHDCA nanoparticles (20 μg/ml). Non-treated control cells (a), 10-min pre-treatment with 3 μg/ml filipin (b), 3 μg/ml nystatin (c), 30 μM chlorpromazine (d) and 0.1% sodium azide (e). Bar represents 20 μm.

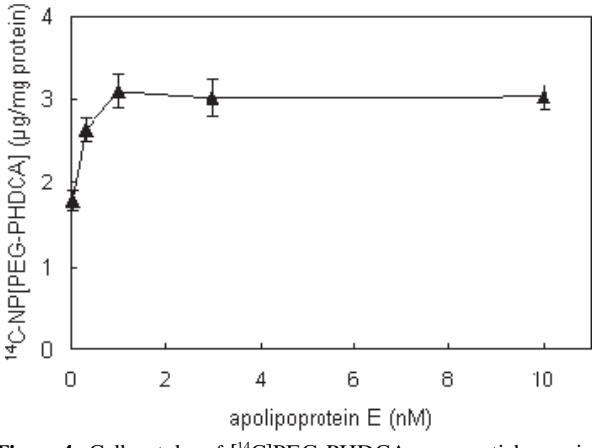

Figure 4. Cell uptake of [^{14}C]PEG-PHDCA nanoparticles preincubated with different concentrations of apolipoprotein E (ApoE).

Two different mechanisms might be responsible for the vesicle-mediated uptake of PEG-PHDCA nanoparticles: clathrin-coated pits (vesicles of 100–150 nm), the ubiquitous classical route of vesicle-mediated endocytosis, and caveolae (50–80 nm), one type of vesicles that is not dependent on the clathrin-mediated pathway [35]. Proteins involved in these vesicle-mediated endocytosis systems, LDLR, LRP-1 and caveolin-1 were detected in RBEC by Western blotting (Fig. 2). In inhibition studies, filipin and nystatin, drugs that bind sterols and disrupt caveolae, were found not to reduce PEG-PHDCA nanoparticle uptake (Fig. 3b, c). Thus, caveolae seem not to be responsible for nanoparticle internalization by RBEC. Furthermore, the cell fractionation results (Fig. 1b) were consistent with a caveolae-independent

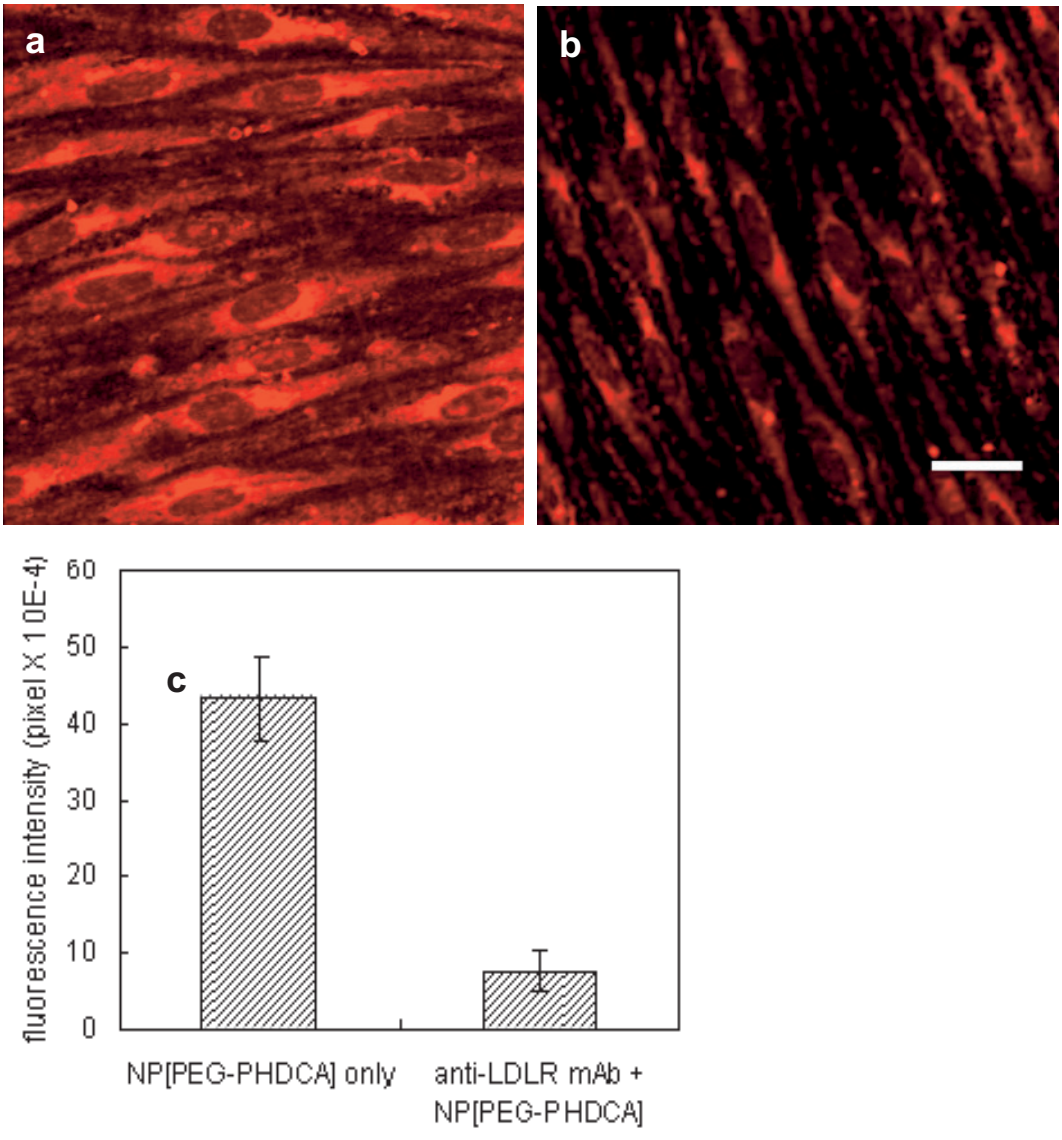

Figure 5. Confocal microscopy observations of RBEC after incubation (20 min; 37°C) of 20 μg/ml nile red fluorescent PEG-PHDCA nanoparticles. Control non-pretreated cells (a) and 1-h pre-treatment with 2.5 μg/ml anti-LDLR mAb as described in the Materials and methods section (b). Bar represents 20 μm. Fluorescence images were quantified by the 'Image J' computer program for three independent experiments (c).

pathway, because the 1% Triton insoluble fraction that would contain the caveolae did not change as a function of time.

Clathrins are mainly found in small invaginations in the cell membrane called coated pits that pinch off from the cell surface to form coated endocytic vesicles [36]. The formation of clathrin-coated pits is generally linked to the action of proteins from the LDLR gene family, including LDLR and LRP-1, when carrying external ligands into cells by receptor-mediated endocytosis. In particular, ligand recognition by the LDLR mobilizes clathrin-coated pit assembly in the cell membrane for internalization. The involvement of this pathway was tested by pretreatment with chlorpromazine (Fig. 3d), a cationic amphiphilic drug that inhibits receptor recycling and prevents clathrin-coated pit assembly. This pretreatment, and also pretreatment with NaN_3 (Fig. 3e), an inhibitor of energy-dependent receptor-mediated pathways, remarkably reduced the amount of fluorescent PEG-PHDCA nanoparticles internalized by RBEC. These data clearly demonstrate that the uptake of the PEG-PHDCA nanoparticles by these cells is due to clathrin-dependent specific receptor-mediated endocytosis.

ApoE is a ligand for the LDLR gene family in the brain [37]; it binds to receptors on the BBB such as LDLR, LRP-1 [38], which were detected by Western blotting in our model (Fig. 2), very low density lipoprotein receptor (VLDLR) [39, 40], apolipoprotein receptor-2 (ApoER-2) [41, 42] and megalin/gp330 [43] as well as receptors in other parts of the central nervous system. ApoE participates in the transport of

lipids into the brain by the LDLR to maintain cholesterol homeostasis [44]. The uptake of PEG-PHDCA nanoparticles preincubated with ApoE by RBEC increased in a concentration-dependent manner (Fig. 4), suggesting that PEG-PHDCA nanoparticles enter RBEC through receptor-mediated endocytosis triggered by the recognition of ApoE adsorbed onto the nanoparticle surface.

LRP-1 is a multifunctional receptor that binds over 30 different ligands [45], and is not only involved in lipid metabolism but also in signal transduction for the activation of Src family kinases [46] and α2-macroglobulin receptor which removes many extracellular proteases and proteinase complexes [47]. Furthermore, VLDLR and ApoER2 have been found to be essential components of a developmental signaling pathway that regulates the lamination of the cortical layers in the brain and involves the activation of tyrosine kinases [48]. On the other hand, LDLR plays an important role in cholesterol homeostasis through removal of low density lipoprotein (LDL) particles from the circulation. Despite extensive studies of over 600 naturally occurring functional mutations, other functions have not been reported. In addition, endocytosis through clathrin-coated pits is essential for the cellular uptake of LDL by LDLR [49, 50] in which the cytoplasm domain of the LDLR is responsible for inducing pit assembly [51]. Hence, the implication of LDLR in the uptake of PEG-PHDCA nanoparticles by RBEC was examined. The reduction observed in the presence of anti-LDLR mAb (Fig. 5), which masks the ligand-binding domain of LDLR, clearly demonstrated that the LDLR was involved in the internalization of PEG-PHDCA nanoparticles.

In conclusion, the intracellular trafficking and confocal microscopy experiments described here have clearly demonstrated that PEG-PHDCA nanoparticles were captured by RBEC through endocytosis. The inhibition studies have shown that these nanoparticles were internalized by the clathrin-coated pit-dependent receptor-mediated pathway. By blocking the ligand-binding domain of LDLR using anti-LDLR mAb and by preincubating the nanoparticles with ApoE, we demonstrated that the uptake of PEG-PHDCA nanoparticles occurred through LDLR-mediated endocytosis in RBEC. Taken together, these results suggest that the LDLR could recognize the nanoparticles at the external face of the cell membrane, after which LDLR-nanoparticles complexes were internalized via clathrin-coated pits formation. In the subsequent intracellular traffic, the LDLR would be recycled and the nanoparticles would accumulate in the endosomal/lysosomal compartments. This study therefore increases our understanding of the brain translocation mechanism of PEG-PHDCA nanoparticles. The specific recognition by LDLR of PEG-PHDCA nanoparticles seems to be due to the distinct adsorption profile of plasma protein onto the nanoparticle surface. A study of this adsorption is now in progress.

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