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Delivery of negatively charged liposomes into the atherosclerotic plaque of apolipoprotein E-deficient mouse aortic tissue

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Abstract

Liposomes have been used to diagnose and treat cancer and, to a lesser extent, cardiovascular disease. We previously showed the uptake of anionic liposomes into the atheromas of Watanabe heritable hyperlipidemic rabbits within lipid pools. However, the cellular distribution of anionic liposomes in atherosclerotic plaque remains undescribed. In addition, how anionic liposomes are absorbed into atherosclerotic plaque is unclear. We investigated the uptake and distribution of anionic liposomes in atherosclerotic plaque in aortic tissues from apolipoprotein E-deficient (ApoE^{-/-}) mice. To facilitate the tracking of liposomes, we used liposomes containing fluorescently labeled non-silencing small interfering RNA. Confocal microscopy analysis showed the uptake of anionic liposomes into atherosclerotic plaque and colocalization with macrophages. Transmission electron microscopy analysis revealed anionic liposomal accumulation in macrophages. To investigate how anionic liposomes cross the local endothelial barrier, we examined the role of clathrin-mediated endocytosis in human coronary artery endothelial cells (HCAECs) treated with or without the inflammatory cytokine tumor necrosis factor (TNF)- α . Pretreatment with amantadine, an inhibitor of clathrin-mediated endocytosis, significantly decreased liposomal uptake in HCAECs treated with or without TNF- α by 77% and 46%,

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Declaration of interest

The authors have no conflicts of interest.

respectively. Immunoblot analysis showed that endogenous clathrin expression was significantly increased in HCAECs stimulated with TNF- α but was inhibited by amantadine. These studies indicated that clathrin-mediated endocytosis is partly responsible for the uptake of liposomes by endothelial cells. Our results suggest that anionic liposomes target macrophage-rich areas of vulnerable plaque in ApoE^{-/-} mice; this finding may lead to the development of novel diagnostic and therapeutic strategies for treating vulnerable plaque in humans.

Keywords

Atherosclerosis; endocytosis; human coronary artery endothelial cells; macrophage

Introduction

Atherosclerosis, a chronic inflammatory disease, is the principal cause of heart attack and stroke and is responsible for 30% of all deaths in the United States (Hansson & Libby, 2006; Ross, 1993; Yusuf et al., 2001). Atherosclerosis is characterized as a systemic, progressive disease process in which the arterial wall thickens through a process of inflammation (Fuster et al., 2005; Granada et al., 2004; Jefferson & Topol, 2005; Ross, 1999b), oxidative stress and dyslipidemia (Quinn et al., 1987; Stocker & Keaney, 2004; Wang & Bennett, 2012). This process leads to plaque formation and flow limitation in the vessel lumen. Early and intermediate-stage atherosclerotic plaque is characterized by thin fibrous caps, lipid-laden cores, inflammation and endothelial dysfunction (Libby, 2002; Naghavi et al., 2003). As a result, local vascular permeability increases due to alterations in endothelial cell endocytosis (Sluimer et al., 2009), damage to the glycocalyx layer (Salmon et al., 2009) and the loosening of gap junctions between cells (Sluimer et al., 2009). The increase in vascular permeability leads to increases in paracellular transport of macromolecules and endothelial cell endocytosis. Similar alterations of vascular permeability occur during tumor growth in cancers (Kisseleva et al., 2006). These increases in vascular and endothelial cell permeability may provide favorable conditions for the localized delivery of therapeutic agents to treat cancer and atherosclerosis.

Liposomes have been used in the diagnosis and treatment of cancer (Chang et al., 2009; Mangala et al., 2009) and, to a lesser extent, cardiovascular disease (Hamilton et al., 2004; Landen et al., 2005; Walton et al., 2010). Because liposomes can be manipulated, their size and external charge can be specified. The aqueous cores of liposomes have the ability to concentrate hydrophilic compounds for imaging (Lopez-Berestein, 1987, 1989; Lopez-Berestein et al., 1984b). Liposomes are also considered less toxic than nonbiologic nanoparticles. Moreover, liposomes have been used successfully in molecular imaging such as single photon emission computed tomography (Lopez-Berestein, 1987, 1989; Lopez-Berestein et al., 1984b) and as nuclear isotope imaging agents to enhance local tissue imaging (Lopez-Berestein et al., 1984a). Therefore, if liposomes are capable of targeting select tissues and delivering sufficient quantities for imaging, then their malleable nature may allow for the local delivery of novel therapeutic compounds to atherosclerotic plaque.

Liposome uptake by tumors is speculated to be the result of altered vascular permeability; however, no definitive mechanism has been described. We have previously demonstrated the uptake of anionic liposomes into the atheromas of Watanabe heritable hyperlipidemic rabbits within lipid pools, suggesting that anionic liposome uptake is associated with areas of metabolically active plaque (Walton et al., 2010). Macrophages are the primary metabolically active component of atherosclerotic plaque (Bhargava & Lee, 2012; Feig & Feig, 2012). In addition, the strong negative charge of anionic liposomes may lead to the preferential phagocytosis of these liposomes by macrophages. The uptake of anionic liposomes into atherosclerotic plaque requires that the liposomes cross the local endothelial barrier; however, the mechanism by which anionic liposomes are absorbed by endothelial cells is not understood. In a previous unpublished study conducted in domestic swine, we found that endothelial dysfunction that results in altered permeability may not alone be sufficient to facilitate the endothelial absorption of anionic liposomes, indicating that most anionic liposome uptake occurs through other pathways.

Apolipoprotein E (ApoE) is an essential protein for the transport and metabolism of lipids. The lack of ApoE results in the rapid development of atherosclerotic lesions that are similar to those that occur in human atherosclerosis. The *ApoE*^{-/-} mouse is a well-validated model for studying human atherosclerosis. These mice, which have a targeted deletion in the *ApoE* gene, develop severe hypercholesterolemia and atherosclerotic lesions in the aortic root and throughout the aorta in a reproducible, time-dependent manner (Breslow, 1996; Nakashima et al., 1994; Piedrahita et al., 1992; Plump et al., 1992; Zaragoza et al., 2011; Zhang et al., 1992). Importantly, *ApoE*^{-/-} mice are the only genetically engineered mice that develop extensive atherosclerotic lesions while consuming a chow diet, and they are the mice in which these lesions have been characterized most thoroughly (Jawien, 2012). In our study, we investigated the uptake and distribution of anionic liposomes in atherosclerotic plaque in aortic tissues from *ApoE*^{-/-} mice. Furthermore, using human coronary artery endothelial cells (HCAECs), we characterized the mechanism by which anionic liposomes are absorbed by endothelial cells under normal and tumor necrosis factor (TNF- α)-stimulated inflammatory conditions.

Methods

Liposome preparation

Liposomes were prepared with 1,2-dimyristoyl-sn-glycero-3-phosphocholine and 1,2-dimyristoyl-sn-glycero-3-phospho-glycerol (Avanti Polar Lipids, Inc., Alabaster, AL) in a 7:3 molar ratio. Lipids and a non-silencing small interfering RNA (siRNA) sequence tagged with Alexa Fluor 488 dye (Qiagen, Valencia, CA) were mixed in excess tertiary butanol, and Tween 20 was added. This mixture was then lyophilized and stored at -20 °C until use. Before *in vivo* administration, this preparation was hydrated with 1 $\times$ phosphate-buffered saline (PBS) at a concentration of 50 μ g/ml to achieve the desired dose in a 100 μ l injection. A non-silencing siRNA sequence tagged with Alexa Fluor 488 (green), which does not share any sequence homology with any known human mRNA sequences, was incorporated into the liposomes and used as a label for the identification and quantification of uptake and distribution in tissues *in vivo*. Preliminary experiments were performed to determine that

liposomal-encapsulated siRNA was efficient for *in vivo* siRNA delivery, which is consistent with findings from a previous study showing that the possibility of siRNA leakage is very low (Landen et al., 2005).

Animal procedure and delivery of liposomes

ApoE^{-/-} mice were purchased from The Jackson Laboratory (Bar Harbor, ME). The animal experimental protocol was performed in compliance with institutional guidelines and was approved by the Animal Welfare Committee (Permit number: AWC 11-069) at The University of Texas Health Science Center at Houston. Eleven *ApoE*^{-/-} mice (9–11 weeks old, C57BL/6 background) were randomly divided into the control group (*n* = 5) and test group (*n* = 6). The mice were fed a normal chow diet. The mice were anesthetized with 2% isoflurane and placed in dorsal recumbency. A topical hair removal cream was applied to the femoral area and rinsed off once the hair was removed. Topical lidocaine was applied to the skin over the femoral vein as a vasodilator. A small incision was made to allow visualization of the femoral vein. A slow injection of 100 µL PBS (control group) or liposomes (5 µg in 100 µL PBS; test group) was administered. A small amount of GLUture (Abbott Laboratories, Abbott Park, IL) was applied to the incision for closure. While still anesthetized, the mice were euthanized by exsanguination *via* cardiac puncture 30 or 60 min (*n* = 3 per group) later for the test group and 60 min (*n* = 5) later for the control group. The aorta of each mouse was collected for immunofluorescence staining and transmission electron microscopy (TEM) analyses.

Tissue processing and atherosclerotic lesion detection

Aortas were divided into two sections. One section was fixed for immunofluorescence staining in 4% paraformaldehyde in PBS (pH 7.4) overnight at 4 °C. The tissue was then rinsed in 15% sucrose for 24 h at 4 °C. The plaque tissues were identified, cut into 5-mm segments, embedded in optimal cutting temperature (OCT) compound (Tissue-Tek; Sakura Finetek, Torrance, CA) and stored at -80 °C before slides were made. The second section of tissue was fixed for TEM analysis in 3% glutaraldehyde in PBS (pH 7.4). Slides containing 5–8 µm of thin sections of OCT-embedded *ApoE*^{-/-} mouse aortic tissues were cut with a Leica CM1800 Cryostat (Leica, Bensheim, Germany), placed on Fisherbrand #15 Superfrost Plus positively charged slides and stored at -80 °C overnight. For assessment of the atherosclerotic lesions, the slides were stained with Oil Red O (Sigma-Aldrich, St. Louis, MO), and images were observed with an Olympus IX71 brightfield microscope (Tokyo, Japan).

Confocal fluorescence microscopy

To determine the uptake and distribution of anionic liposomes in atherosclerotic plaque in *ApoE*^{-/-} mouse aortic tissue, the slides were washed with PBS and counterstained with 4', 6'-diamidino-2-phenylindole (DAPI) nucleic acid stain. Images were observed on a Leica SP5 confocal system with a Leica TCS SP5 II microscope (Leica Microsystems, Mannheim, Germany), DAPI and fluorescein isothiocyanate (FITC) filters and LAS AF image acquisition software (Leica Microsystems, Mannheim, Germany). For colocalization analysis, the slides were incubated in 1% bovine serum albumin (BSA)/10% normal goat

serum in 0.1% PBS-Tween 20 for 1 h to permeabilize the tissue and block nonspecific protein–protein interactions. The slides were then incubated with rabbit polyclonal anti-CD68 (sc-9139; Santa Cruz Biotechnology, Santa Cruz, CA; 1:100 dilution) at 4 °C overnight, washed three times with 1 × PBS and incubated with anti-rabbit Texas red conjugated secondary antibody (#6719, Abcam, Cambridge, UK; 1:1000 dilution) at room temperature for 60 min. Images were analyzed on a Leica SP5 confocal system with a Leica TCS SP5 II microscope as described above. To quantify the uptake of liposomal siRNA by macrophages relative to that by other cells in atherosclerotic plaque, we calculated the percent of the ratio of the number of liposomal siRNA puncta that colocalized with macrophages over the total number of liposomal siRNA puncta in plaques. For each group, five random visual fields were analyzed.

Transmission electron microscopy

To further characterize the distribution of liposomes in atherosclerotic plaque, tissue sections were initially fixed with 3% glutaraldehyde in PBS (pH 7.4) and post-fixed in 1% osmium tetroxide. Dehydration was carried out in a series of graded alcohol washes (70%, 80%, 90% and 100% ethanol), followed by two acetone washes. The samples were infiltrated with Epon plastic resin, embedded and cut into 1 µm-thick sections with an RMC MTXL Ultra Microtome (Boeckeler Instruments, Tucson, AZ) for low-resolution images. Furthermore, the plaque sections were cut into 60–80 nm-thick sections for ultra-structure images. The thin sections were stained for uranyl acetate and lead citrate. Images were acquired with a JEOL JeM-1230 transmission electron microscope (JEOL, Tokyo, Japan).

Cell culture and treatment

HCAECs were purchased from Lonza Walkersville, Inc. (Walkersville, MD). Cells were cultured in EBM-2 (Lonza) medium. Liposomes were added to serum-free incubation medium at a final concentration of 150 µg/ml total lipids. To measure liposome uptake, medium was replaced by liposome-containing medium or medium without liposomes (control) and incubated for 30 min. In the TNF-α treatment experiment, cells were incubated with TNF-α (Research and Diagnostic System, 100 ng/ml) for one hour to analyze clathrin expression. Then, the cells were washed and incubated with liposomes. To evaluate the uptake of liposomes by the clathrin-mediated pathway, cells were pretreated with 1 mM amantadine (Sigma-Aldrich) for 20 min before incubation with TNF-α, as described above. To evaluate the effects of amantadine on clathrin expression, cells treated with TNF-α or pretreated with amantadine followed by TNF-α were incubated with rabbit anti-clathrin primary antibody and anti-rabbit FITC-conjugated secondary antibody (#6717, Abcam, 1:1000 dilution). The uptake of fluorescent liposomes and clathrin expression were visualized by using a Leica SP5 confocal system with a Leica TCS SP5 II microscope as described above. For the quantitative analysis of fluorescence, cells were trypsinized, resuspended in ice-cold PBS and analyzed by using a BD LSR II flow cytometer (BD Biosciences, Franklin Lakes, NJ) with a blue laser (488 nm). Automated compensation was performed by using FACSDiva Version 6.1.3 software (BD BioSciences, San Jose, CA).

Immunoblotting assay

To prepare total cellular extracts, cells were isolated after treatment and sonicated in radioimmunoprecipitation assay (RIPA) solution (Thermo Scientific, Rockford, IL). Protein concentrations were measured by using the BCA protein assay reagent (Pierce, Rockford, IL). Proteins were separated by using 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (25 µg protein/lane) and transferred to Immun-Blot PVDF membrane/filter paper (Bio-Rad, Hercules, CA), which was then incubated with polyclonal anti-clathrin heavy-chain antibody (#2410, Cell Signaling Technology Inc., Danvers, MA). Subsequently, the blot was incubated with goat anti-rabbit horse radish peroxidase-labeled secondary antibody (Bio-Rad). Proteins were detected by using Pierce enhanced chemiluminescence Western blotting substrate. β -Actin (A1978, Sigma-Aldrich) was used as loading control.

Statistical analysis

Data are representative of three experiments or replicates and are expressed as the mean $\pm$ standard error. Experimental groups were compared by using a Student *t* test. A *p* value of less than 0.05 was considered statistically significant.

Results

Liposomal uptake in atherosclerotic plaque

Light microscopy analysis of Oil Red O-stained atherosclerotic plaque (Figure 1A) revealed highly complex lesions. Furthermore, TEM analysis of atherosclerotic plaque showed the presence of foam cells and cholesterol monohydrate crystals (Figure 1B and C). To examine the uptake and distribution of anionic liposomes in atherosclerotic plaque in the aortic tissue of *ApoE*^{-/-} mice, we performed immunofluorescence staining after the injection of PBS (Figure 2A) or fluorescently labeled liposomes (Figure 2B and C). At 30 min (Figure 2B) and 60 min (Figure 2C) after injection, a significant amount of fluorescently labeled liposome was seen in the atherosclerotic plaque of all *ApoE*^{-/-} mice, whereas no fluorescence was detected in atherosclerotic plaque after the injection of PBS (Figure 2A). Liposomes were most abundant in lipid-rich areas of atherosclerotic plaque, as demonstrated by the absence of DAPI staining in these regions.

Colocalization of macrophages and fluorescently labeled liposomes in atherosclerotic plaque

It is generally believed that metabolically active components of atherosclerotic plaque, such as macrophages, preferentially uptake negatively charged particles. To determine whether atherosclerosis-associated macrophages expressing CD68 colocalized with fluorescently labeled liposomes in atherosclerotic plaque, we performed confocal fluorescence imaging of atherosclerotic plaque in the aortic tissue of *ApoE*^{-/-} mice. CD68 staining indicated the presence of macrophages in the plaque area, and fluorescently labeled liposomes were visible in the macrophage-rich area of plaque (Figure 3A and B). Fluorescently labeled liposomes and CD68+ macrophages colocalized primarily in the adventitia lipid-rich areas of plaque (Figure 3A–C). Quantitative analysis showed that approximately 70% of liposomal siRNA uptake in plaque colocalized with macrophages. To further confirm the

distribution of anionic liposomes, we conducted TEM analysis. As shown in Figure 3D, liposomes accumulated within macrophages in atherosclerotic plaque, suggesting an association between liposomal uptake and areas of metabolically active plaque.

The role of clathrin-mediated endocytosis in liposome uptake by HCAECs

To evaluate whether clathrin-mediated endocytosis is the pathway responsible for the uptake of liposomes by HCAECs, we studied the effects of amantadine, an independent inhibitor of clathrin-mediated endocytosis (Eckels et al., 2009; Luttrell et al., 1999) on liposome uptake in HCAECs. In addition, to examine the effects of amantadine under inflammatory conditions, we treated HCAECs with TNF- α , a key cytokine in the recruitment and activation of inflammatory cells. We first examined the cytotoxicity of amantadine and found that a concentration of 1 mM amantadine was not toxic to HCAECs (<5% of cell death; data not shown); thus, this concentration was used in subsequent experiments. Confocal microscopy analysis showed that the uptake of liposomes was greater in HCAECs treated with TNF- α for one hour than in untreated HCAECs (liposomes only) (Figure 4A). Importantly, the pretreatment of HCAECs with amantadine reduced the uptake of liposomes in both TNF- α -treated or untreated HCAECs (Figure 4A). The quantification of fluorescently labeled liposomes by flow cytometry (expressed as the % of viable cells) showed that the uptake of liposomes by HCAECs was 3.5-fold higher in HCAECs treated with TNF- α for one hour than in untreated HCAECs (liposomes only) (Figure 4B). Furthermore, in untreated HCAECs or HCAECs stimulated with TNF- α , pretreatment with amantadine significantly reduced liposome uptake by 46% and 77%, respectively (Figure 4B). These results suggest that clathrin-mediated endocytosis is at least partially responsible for the uptake of liposomes by HCAECs.

Analysis of clathrin expression in HCAECs

We examined whether the TNF- α -induced increase in the uptake of liposomes was mediated by an increase in clathrin expression. Confocal microscopy analysis showed that the basal expression of endogenous clathrin in HCAECs was low (Figure 5A). However, in cells stimulated with TNF- α for one hour, clathrin expression was significantly increased (Figure 5B). Importantly, amantadine greatly inhibited clathrin expression in TNF- α -stimulated cells (Figure 5C). The results of immunoblot analysis were fully consistent with the results of confocal microscopy (Figure 5D). Together, our data indicate that the TNF- α -induced increase in liposome uptake was mediated by clathrin-dependent endocytosis.

Discussion

In this study, we showed the uptake of anionic liposomes into atherosclerotic plaque in the aortic tissue of *ApoE*^{-/-} mice and that these liposomes colocalize with and accumulate in macrophages. In addition, we showed that the uptake of anionic liposomes into HCAECs occurs through clathrin-mediated endocytosis. Macrophages play a pivotal role in plaque instability, which is directly involved in triggering acute coronary syndromes including unstable angina, acute myocardial infarction and sudden coronary death (Gawaz et al., 2005; Libby, 2001; Parthasarathy et al., 1986; Ross, 1999a; Weber et al., 2008). Thus, our findings suggest that the liposomal drug delivery system may be a promising therapeutic strategy for

targeting the inhibition of macrophage infiltration in atherosclerotic plaque to promote plaque stabilization.

In *ApoE*^{-/-} mice, foam cell lesions develop as early as eight weeks (Nakashima et al., 1994). Similarly, we found that atherosclerotic plaque in the aortas of *ApoE*^{-/-} mice consisted of highly complex lesions and showed evidence of foam cell accumulation. Furthermore, we observed the accumulation of liposomes in macrophages within atherosclerotic plaque, suggesting that the strong negative charge of these liposomes may lead to preferential phagocytosis of the liposomes by macrophages via their scavenger receptors, which in turn followed by conversion into macrophage-derived foam cells and lipid-rich area.

Anionic liposomes have several advantages that positively charged liposomes do not. Namely, positively charged liposomes have been shown to be toxic as a carrier and tend to attach to arterial walls (Filion & Phillips, 1997; Lonez et al., 2008). In contrast, anionic liposomes are passively absorbed through atherosclerotic areas of the arterial wall as a result of altered permeability caused by glycocalyx loss. For liposomes to be taken up into atherosclerotic plaque, they must first cross the local endothelial barrier. Clathrin- and caveolar-mediated endocytosis are the most commonly reported processes of cellular uptake (Brodsky et al., 2001; Filion & Phillips, 1997; Lonez et al., 2008; Mellman, 1996; Pelkmans & Helenius, 2002). Clathrin-coated vesicles are the best characterized of the vesicular carriers and provide a specific delivery system for the uptake of extracellular material (McMahon & Boucrot, 2011). In this study, we showed that the uptake of liposomes into HCAECs was low under normal conditions but was significantly increased in HCAECs stimulated with TNF- α . Furthermore, amantadine, an independent inhibitor of clathrin-mediated endocytosis, significantly inhibited the uptake of liposomes. In addition, our immunoblot analysis showed that the endogenous expression of clathrin was low in HCAECs, but that TNF- α stimulation significantly increased the expression of clathrin; this increase was inhibited by amantadine. These studies indicate that the clathrin-mediated pathway is at least partially responsible for the endocytosis of liposomes by endothelial cells. Furthermore, these results suggest that the presence of atherosclerosis, including the inflammation response, is essential for the increased uptake of liposomes into atherosclerotic plaque. However, because the inhibition of endocytosis by amantadine was incomplete (77% inhibition in TNF- α -treated HCAECs), some liposome uptake may occur through one or more other pathways. Interestingly, our unpublished data showed that the endogenous expression of HCAEC caveolin-1 is higher than that of clathrin, suggesting that caveolar-mediated endocytosis may also be involved in the uptake of liposomes by HCAECs.

Conclusion

Anionic liposomes appear to specifically target the macrophage-rich area of atherosclerotic plaque in the aortic tissue of *ApoE*^{-/-} mice. Furthermore, the uptake of liposomes by local endothelial cells occurs partially through a clathrin-mediated pathway. The targeted delivery of modified liposomes to the macrophage-rich regions of vulnerable plaque may have diagnostic and therapeutic potential in the treatment of vulnerable plaque.

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Figure 1.

Atherosclerotic lesions in *ApoE*^{-/-} mice. (A) Light microscopy image showing Oil Red O staining of an area of atherosclerotic lesions in *ApoE*^{-/-} mouse aortic tissue (20 × magnification). (B and C) TEM images showing foam cell lesions in atherosclerotic plaque (4000 × and 10 000× magnification, respectively). Scale bars = 10 μm in (B) and 2 μm in (C). MFC, macrophage-derived foam cells; CC, cholesterol monohydrate crystal; P, plaque; L, lumen.

Figure 2.

Uptake of anionic liposomes into atherosclerotic plaque in *ApoE*^{-/-} mouse aortic tissue. A nonsilencing siRNA sequence tagged with Alexa Fluor 488 dye (excitation, 495 nm; emission, 519 nm) was incorporated into liposomes and used to determine liposome uptake and distribution in atherosclerotic plaque of aortic tissue. Confocal microscopy images (63 × magnification) showing (A) the absence of fluorescence in atherosclerotic plaque of a PBS-injected control mouse and (B and C) the uptake of fluorescently labeled liposomes into atherosclerotic plaque 30 min (B) and 60 min (C) after the injection of liposomes, seen as green punctate structures. Tissue sections were counterstained with 4',6'-diamidino-2-phenylindole (DAPI) nucleic acid stain. Scale bars = 50 μm L, lumen; P, plaque.

Figure 3.

Distribution of anionic liposomes in the atherosclerotic plaque of *ApoE*^{-/-} mouse aortic tissue. Confocal microscopy images (63 × magnification) showing fluorescently labeled liposomes (A, green) and immunofluorescence staining for CD68+ macrophages (B, red) in atherosclerotic plaque. Liposomes accumulated in macrophage-rich areas. (C) Overlay (yellow) of images depicted in (A) and (B) showing the colocalization of liposomes with macrophages. (D) TEM analysis (20 000 × magnification) showing the accumulation of macrophages in atherosclerotic plaque. Scale bar = 2 μm. Li, liposome; N, nucleus.

Figure 4.

Effect of amantadine on the uptake of fluorescently labeled liposomes into HCAECs. (A) Confocal microscopy analysis ($63 \times$ magnification) showing the uptake of fluorescently labeled liposomes into HCAECs after 30 min of incubation. As indicated, some cells were pretreated with amantadine for 20 min and/or TNF- α for 1 h. (B) Results of flow cytometry analysis showing the quantification of fluorescently labeled liposome uptake in HCAECs. As indicated, some cells were pretreated with amantadine for 20 min or TNF- α for 1 h. Bars represent the mean $\pm$ standard error ($n = 3$; $*p < 0.05$).

Figure 5.

Amantadine inhibits TNF- α induced clathrin overexpression. Confocal microscopy analysis ($63 \times$ magnification) showing clathrin expression (green punctuate structures) in (A) untreated HCAECs, (B) HCAECs pretreated with TNF- α for 1 h and (C) HCAECs pretreated with 1 mM amantadine for 20 min and TNF- α for 1 h. (D) Immunoblot analysis showing results similar to those above. β -actin was used as loading control. HC, heavy chain; Amt, amantadine.