Hypoxic Induction of Exosome Uptake through Proteoglycan-Dependent Endocytosis Fuels the Lipid Droplet Phenotype in Glioma

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ABSTRACT

As an adaptive response to hypoxic stress, aggressive tumors rewire their metabolic phenotype into increased malignant behavior through extracellular lipid scavenging and storage in lipid droplets (LD). However, the underlying mechanisms and potential lipid source retrieved in the hypoxic tumor microenvironment remain poorly understood. Here, we show that exosome-like extracellular vesicles (EV), known as influential messengers in the tumor microenvironment, may also serve anabolic functions by transforming hypoxic, patient-derived human glioblastoma cell lines into the LD phenotype. EVs were internalized via a hypoxia-sensitive, endocytic mechanism that fueled LD formation through direct lipid transfer, and independently of fatty acid synthase activity.

Introduction

Tumor aggressiveness is associated with metabolic adaptation that rescues tumor cells from hypoxic stress and attenuates the cytotoxic effect of oncologic treatment. Lipid accumulation in the form of lipid droplets (LD) is a central metabolic phenotype that drives the malignant behavior of several tumor types. LD formation promotes tumor progression at the autocrine and paracrine level by, for example, securing nutrient and energy supply, increased survival in the blood stream during metastasis, scavenging of free radicals, and by transforming the tumor microenvironment (1–5). Earlier studies suggested a major role of de novo lipogenesis from increased utilization of glucose and glutamine carbons and autocrine processes. However, more recent work has demonstrated the requirement of increased scavenging of extracellular lipids, a process considered less energy consuming than endogenous lipid biosynthesis (6–10). Although the promalignant effects of LD loading are well established, it remains an important question how poorly perfused aggressive tumors can retrieve lipids from their surroundings. The LD phenotype has been specifically linked to extreme metabolic stress of the hypoxic tumor niche, distinguished by impaired lipolysis and mitochondrial oxidative capacity, and that is relatively inaccessible to systemic supply of lipids. This is especially true for high-grade gliomas (HGG), including glioblastoma, that is, the most common and aggressive type of primary brain tumor that resides in a location with limited access to oxygen and circulating nutrients (11–13). Extracellular vesicles (EV), including exosomes and microvesicles, are known as paracrine signalosomes that can elicit profound effects in target cells (14, 15). EVs have been implicated in the metabolic adaptation that occurs in the tumor microenvironment by exchanging metabolites encapsulated in their lumen (16). EV-mediated protumorigenic effects in glioma are well documented and generally assumed to depend on intercellular transfer of EV-associated miRNAs and signaling proteins (17–19). Notably, EVs are lipid-rich particles, mainly derived from membrane lipid rafts enriched in cholesterol and glycosphingolipids, and can carry most apolipoproteins classes found on lipoproteins, that is, the main lipid source in the circulation (14, 20). Moreover, EVs have been shown to cross the blood–brain barrier, suggesting that in addition to spreading by local diffusion, EVs can reach their target tissue from distant sites (21).

While there has been a focus on the composition, function, and biogenesis of EVs, the mechanisms of EV cargo transfer to target cells and the intracellular fate of internalized EVs are still poorly defined (15, 22, 23). Numerous reports have shown an increased release of EVs, as well as altered EV composition with potent functional effects in response to tumor hypoxia, pointing at an important role of EVs during hypoxic adaptation (19, 24, 25). However, whether this coincides with increased EV uptake by hypoxic target cells, and whether tumor cell–derived EVs can feed the hypoxic niche to trigger the LD phenotype remain unknown.

Here, we were interested in elucidating whether EVs may contribute to metabolic reprogramming of hypoxic tumor cells, and to better understand how hypoxia regulates tumor cell recruitment and processing of EVs. Our data indicate that tumor cell–derived EVs can convert hypoxic HGG cells into the LD phenotype by direct lipid transfer, in a
manner comparable with whole-serum lipids and lipoproteins. These findings expand our view of the protumorigenic activities of EVs with possible implications for EV-directed targeting strategies in cancer.

Materials and Methods

Cells and culture conditions

U87 MG human HGG (ATCC; HTB-14, RRID:CVCL_0022) and GL261 mouse HGG cells (ref. 26; RRID:CVCL_Y003, generously provided by Dr. Anna Darabi, Lund University, Lund, Sweden) were cultured in DMEM with high glucose (HyClone, GE Healthcare), supplemented with 10% (volume/volume) FBS (Sigma-Aldrich), 2 mmol/L l-glutamine, 100 U/mL penicillin, and 100 μg/mL Streptomycin (Thermo Fisher Scientific; full medium) for >30 passages. For cell authentication, ATCC uses morphology, karyotyping, cytochrome C oxidase I (COI) analysis, and short tandem repeat (STR) profiling. GL261 cells were authenticated by us (in 2016), by their typical glioblastoma tumor morphology when injected into the brains of syngeneic C57BL/6J mice. Primary, patient-derived human glioblastoma cell lines, U3034, U3065, and U3082, were from the Human Glioblastoma Cell Culture resource (www.hgcc.se; RRIDs: CVCL_IR73, CVCL_IR87 and CVCL_IR93), authenticated by STR profiling (27), and cultured on poly-L-ornithine- and laminin-coated surfaces in serum-free neurobasal:F12 (1:1) medium supplemented with EGF, FGF, and stem cell supplements, as described previously (27) for <10 passages. All cells were routinely grown in a humidified 21% O2 and 5% CO2 incubator at 37°C and tested for Mycoplasma by DAPI staining and high-resolution confocal microscopy at least once a month. For hypoxia experiments, cells were incubated in a humidified hypoxia station (SCI-TIVE N-N, Baker Ruskinn) set at 1% O2, 5% CO2, 94% N2, and 10% CO2 for 6 hours in serum-free medium, and then incubated with PKH-labeled EVs and either of cholera toxin subunit B-AF488 (CtxB; 25 μg/mL; Thermo Fisher Scientific; C34775), dextran-FITC (Dex; 2.5 mg/mL; Sigma-Aldrich; FD10S, 10 kDa), or transferrin-AF488 (Tfn; 10 μg/mL; Invitrogen; T13342) endocytosis ligands for 30 minutes at maintained normoxia or hypoxia. Residual cell surface bound EVs and ligands were eluted with a 1 mol/L NaCl wash, and extensive washing with ice-cold PBS prior to imaging analysis. In SDC1-CtxB-EV colocalization experiments, cells were preincubated with a rabbit anti-human SDC1 antibody (Abcam; ab128936, RRID: AB_11150990, 1:500) on ice for 30 minutes for membrane SDC1 binding, followed by extensive washing with PBS, and incubation with PKH67-EVs and CtxB-AF647 (Thermo Fisher Scientific; C34778) for 30 minutes at 37°C. Cells were washed, fixed, and permeabilized with 0.5% saponin for 15 minutes at room temperature before staining with an anti-rabbit-AF546 antibody (Thermo Fisher Scientific; A-11010, RRID:AB_2534077, 1:500). Confocal microscopy analysis

In EV uptake experiments, EVs were isolated as described above, and after the second centrifugation step, labeled using PKH67 green or PKH26 red Fluorescence Lipophylic Dyes (MID676 and MID126; Sigma-Aldrich), as described previously (29). HGG cells were seeded in chambered slides, preconditioned in normoxia or hypoxia for 6 hours in serum-free medium, and then incubated with PKH-labeled EVs (50 μg/mL, in serum-free medium) for 1 hour at 37°C with maintained normoxia or hypoxia. For colocalization studies, cells preconditioned in hypoxia or normoxia for 6 hours were incubated with PKH-labeled EVs and either of cholera toxin subunit B-AF488 (CtxB; 25 μg/mL; Thermo Fisher Scientific; C34775), dextran-FITC (Dex; 2.5 mg/mL; Sigma-Aldrich; FD10S, 10 kDa), or transferrin-AF488 (Tfn; 10 μg/mL; Invitrogen; T13342) endocytosis ligands for 30 minutes at maintained normoxia or hypoxia. Residual cell surface bound EVs and ligands were eluted with a 1 mol/L NaCl wash, and extensive washing with ice-cold PBS prior to imaging analysis. In SDC1-CtxB-EV colocalization experiments, cells were preincubated with a rabbit anti-human SDC1 antibody (Abcam; ab128936, RRID: AB_11150990, 1:500) on ice for 30 minutes for membrane SDC1 binding, followed by extensive washing with PBS, and incubation with PKH67-EVs and CtxB-AF647 (Thermo Fisher Scientific; C34778) for 30 minutes at 37°C. Cells were washed, fixed, and permeabilized with 0.5% saponin for 15 minutes at room temperature before staining with an anti-rabbit-AF546 antibody (Thermo Fisher Scientific; A-11010, RRID:AB_2534077, 1:500). For staining of membrane lipid rafts, the Vybrant Alexa Fluor 555 Lipid Raft Labeling Kit (Thermo Fisher Scientific; V34404) was used, according to the manufacturer’s instructions. Briefly, nonfixed normoxic or hypoxic cells were incubated with fluorescent CtxB, following washes with serum-free medium, and cross-linking with antibody against CtxB. Membrane cholesterol staining was performed using filipin III (250 μg/mL; F4767; Sigma-Aldrich).

For confocal microscopy analysis, cells were fixed with 4% (w/v) paraformaldehyde for 10 minutes on ice and counterstained for nuclei with Hoechst33342 (1:20,000, 1399; Life Technologies). Confocal images were acquired on a Zeiss LSM710 confocal fluorescence microscope system using laser excitation at 405, 488, or 546 nm and EC Plan-Neofluor 40×/1.30 Oil DIC M27 or EC Plan-Apochromat 63×/1.4 Oil DIC M27 Oil immersion objectives, integrated with the Zen Software (Zeiss, RRID:SCR_018163).

Live-cell imaging of internalized EVs and CtxB was performed with the same microscope equipped with heat incubator set at 37°C with 5%
CO2. Cells were grown in chamber slides and EVs with or without CtxB were added to subconfluent cells in serum-free medium without FBS and incubated for 1 hour. Surface bound ligands were removed by extensive washing with 1 mol/L NaCl and serum-free medium, followed by live-cell imaging of intracellular ligands in phenol red-free medium during time series, as indicated in corresponding video legends.

**Triglyceride content analysis**

U87 MG cells were incubated in normoxia or hypoxia in serum-free medium with or without EVs (50 µg/mL) or LDL (LP2; Merck, 50 µg/mL) or in full medium for 48 hours. Cells were then detached with TriPEX Express (Thermo Fisher Scientific; #12605036) and resuspended in PBS + 1% Triton X-100 at 10^6 cells/mL, followed by analysis using the Serum Triglyceride Quantification Kit (Cell Biolabs; STA-397). Results were normalized to cell number and expressed as µg of triglyceride per 10^6 cells.

**Flow cytometry analysis of ligand internalization and cell surface binding**

HGG cells were preconditioned in normoxia or hypoxia in serum-free medium, as indicated in the respective figure legend, and then incubated at maintained normoxia or hypoxia with PKH-labeled EVs, CtxB, Dx, or Tfn for 1 hour. In some cases, pharmacologic stabilization of hypoxia-inducible factors (HIF) was induced in normoxic cells by 24-hour pretreatment with desferroxamine (Sigma-Aldrich; 125 µmol/L) or dimethylsulfoxycycloheximide (Sigma-Aldrich; 1 mmol/L) in serum-free medium. In membrane cholesterol depletion experiments, cells were treated with methyl-β-cyclodextrin (MCD; Sigma-Aldrich; 2.5 mmol/L (U87 MG cells) or 5 mmol/L (GL261 cells)) during 1 hour prior to and during EV uptake. For membrane cholesterol loading, cholesterol (C8667; Sigma-Aldrich) was complexed with MCD at a ratio of 1:8 (cholesterol:MCD) as described in (31). Briefly, cholesterol (2.5 mmol/L) was thoroughly mixed with MCD until dissolved, and cells were incubated with cholesterol:MCD complexes for 1 hour prior to EV uptake. For anti-HS and anti-chondroitin sulfate (CS) antibody internalization, mouse anti-CS antibody (Sigma-Aldrich; CS-56, #SAB4200696; ref. 32) or single-chain fragment variable (scFv) antibodies against HS (clones HS4C3 and AO4B08; refs. 33, 34) or CS chains (clone GDS87; ref. 35) were precomplexed for 30 minutes at room temperature with mouse anti-tag antibody (Sigma-Aldrich; V5507, RRID:AB_251877; 1:500, for scFv antibodies) and/or anti-mouse-AF488 (Thermo Fisher Scientific; A-11001, RRID:AB_2534069) prior to internalization for 1 hour. Cells were then extensively washed with PBS and 1 mol/L NaCl, detached with trypsin, and washed in PBS prior to analysis.

For EV cell surface binding and staining of cell surface heparan sulfate proteoglycan (HSPG) and CSPG, cells were detached with 2 × PBS containing 0.5 mmol/L EDTA, washed with PBS with Ca^2+ and Mg^2+ (PBS+-/), and blocked with 3% BSA-containing PBS+-/ followed by incubation with PKH-labeled EVs (15 µg/mL) or primary antibodies against HS and CS and fluorescently labeled secondary antibodies. Cells were extensively washed in PBS before analysis. In all the above cases, cellular mean fluorescence intensity (MFI) was acquired on an Accuri C6 Flow Cytometer (BD Biosciences) using a 488-nm wavelength excitation laser and the FL1-H detector (533±50 nm). Three independent wells per condition were analyzed in each experimental run, which was repeated in two or three independent experiments. Data were analyzed using Accuri C6 software. MFI values per sample were averaged after subtraction of background fluorescence (MFI of cells incubated without ligand). After normalization to the respective control samples, results from the different experimental runs were presented as the mean or mean fold ± SD.

**Cell surface HSPG inhibition**

In heparin competition experiments, exogenous full-length heparin (H3339; Sigma-Aldrich) was added at the indicated concentrations together with PKH-labeled EVs in serum-free medium. For HS lyases digestion experiments, cells were cultured in serum-free medium and pretreated with 1.2 mIU/mL Heparinase I (H2519; Sigma-Aldrich) and 0.6 mIU/mL Heparinase III (H8891; Sigma-Aldrich) for 3 hours at 37°C. Enzyme addition was repeated and the incubation was allowed to proceed for another 3 hours, followed by extensive washing and addition of EVs in serum-free medium. Pharmaceutical inhibition of PG biosynthesis was achieved by preincubation with 2.5 mmol/L PNP-sulfate (N2132; Sigma-Aldrich) during 48 hours prior to EV uptake. For HS- and CS-lyase inhibition experiments, cells were pretreated with sodium chlorate (NaClO3, 25 mmol/L; Alfa Aesar, 044408) or NaCl as a control for 24 hours prior to incubation with EVs in serum-free medium. Finally, cells were analyzed by confocal microscopy and/or flow cytometry, as described above.

**HIF silencing and Western blot analysis**

For knockdown (KD) of HIF1α and HIF2α, cells were transfected with 100 nmol/L of nontargeting siRNA (AM4613), HIF1α-specific siRNAs, #1 (#428/40) or #2 (#56359), and HIF2α-specific siRNAs, #1 (#4699) or #2 (#4700; Ambion), using DharmaFECT Transfection Reagent (T-2001-01; Dharmacon). Twenty-four hours posttransfection, cells were reseeded for downstream EV uptake analysis (see above). To validate KD efficiency by Western blot analysis, transfected cells were lysed with RIPA buffer containing Protease Inhibitor Cocktail (cOmplete, 04693124001; Roche). Lysates were clarified by centrifugation at 14,000 × g for 10 minutes, protein concentration was determined by BCA Protein Assay Kit (Pierce), and equal protein amounts were mixed with LDS sample buffer and Reducing Agent (NuPage; Thermo Fisher Scientific), heated at 80°C, and loaded to a 4%–12% NuPage Bis-Tris gel for electrophoresis. Following transfer to polyvinylidene difluoride membranes by electroblotting and blocking with 5% (w/v) BSA, membranes were incubated overnight with primary antibodies at 4°C. Membranes were washed with TBS/TWEEN 0.5% and incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies, for 1 hour at room temperature. HRP was detected using ECL substrate and membranes were developed with chemiluminescence-reactive films. The following antibodies were used: anti-HIF1α (GeneTex; GTX127309, RRID:AB_2616089, 1:1,000); anti-HIF2α (Abcam; ab1999, RRID:AB_302739; 1:750); anti-tubulin (Abcam; ab7291, RRID:AB_2241126; 1:10,000); anti-rabbit HRP (Cell Signaling Technology; #7074, RRID:AB_2099233); and anti-mouse HRP (Sigma-Aldrich; #19044).

**EV lipid transfer experiments**

To explore whether EV-incorporated lipid could be directly processed into LD storage, U87 MG donor cells were loaded with the BODIPY-labeled fatty acid (FA) analogue (BODIPY 500/510 C1, C12, #38323; Thermo Fisher Scientific), previously shown to be metabolized by live cells into natural lipids (36–38), at 5 µmol/L during 48 hours. BODIPY-FA–labeled EVs (hereafter denoted as BODIPY-FA-EV) were then isolated from conditioned medium and separated from free BODIPY-FA by sequential ultracentrifugation (see above). BODIPY-FA-EV internalization was analyzed and compared with PKH-labeled EVs by flow cytometry and confocal microscopy. For lipid transfer experiments, BODIPY-FA-EVs were incubated with
recipient cells over 48 hours, and colocalization with LDs was assessed by LipidTox staining, followed by confocal microscopy (see above).

**Cell viability assay**

Viability of hypoxic U87 MG cells after treatment with FASN inhibitors (Fasnall or GSK2194069; 0–200 μmol/L) for 48 hours was determined by the MTT Assay (Sigma), according to the manufacturer’s recommendations.

**Statistical analysis**

Statistical significance was evaluated with Student two-tailed unpaired t test using Microsoft Excel (RRID:SCR_016137). Sample size (n) was determined by the number of experimental units analyzed (i.e., individual cell culture wells or confocal images) in each of several independent experiments, as indicated in the respective figure legend. Results were normalized to corresponding controls, as indicated, and error bars represent SD, unless otherwise stated. *, P < 0.05 was considered statistically significant.

**Results**

**Exosome-like EVs can induce the LD† phenotype in glioma cells**

EVs derived from the human HGG U87 MG cell line have been comprehensively profiled by our group according to the requirements of the International Society of Extracellular Vesicles (39). EV size and morphology, as assessed by transmission electron microscopy and nanoparticle tracking analysis, showed the typical shape and size distribution of exosomes (50–150 nm in diameter; ref. 28), and RNA analysis revealed a relative depletion of rRNA and enrichment of mRNA and miRNA in EVs versus U87 MG donor cells (19). Moreover, immunoprofiling and untargeted proteomics by LC/MS-MS demonstrated the enrichment of typical exosome markers (e.g., tetraspanins, CD9, CD63, CD81, TSG101, and flotillin-1; refs. 19, 28, 29). Here, we could confirm that U87 MG–derived EVs indeed are relatively enriched in CD9, CD63, and TSG101, and exhibit a size distribution of approximately 50–150 nm (Supplementary Fig. S1A). Initial observations showed an increased accumulation of LDs in the hypoxic niche of U87 MG tumors injected orthotopically in mice, as defined by LD colocalization with the hypoxia response marker, carbonic anhydrase IX (CA9; Fig. 1A). To explore the potential role of EVs, we next compared the LD-inducing capacity of U87 MG cell–derived EVs, full medium (10% FBS), LDL isolated from human serum, and EV/LPDS, as schematically depicted in Fig. 1B. Target glioma cells grown in normoxia displayed few LDs at all conditions tested, whereas hypoxic (1% O2) cells treated with full medium or LDL expectedly acquired the LD† phenotype (Fig. 1C; ref. 40). Hypoxia has been shown to induce FASN and de novo lipid synthesis, concomitantly with reduced lipolysis and mitochondrial oxidative capacity (2, 3). However, hypoxia alone was not sufficient to induce LDs, and medium supplemented with serum deficient in EVs and LPs (EV/LPDS) had no apparent effect on LD formation even at hypoxia (Fig. 1C). Interestingly, we found that EVs can induce the LD† phenotype specifically at hypoxic conditions, as shown by confocal microscopy imaging (Fig. 1D). EV-mediated induction of LD formation in hypoxia was also shown in two different primary human HGG cell cultures (Supplementary Fig. S1B). Quantification of imaging data could confirm a significant LD-inducing effect of EVs in hypoxia that was comparable in magnitude with the effect of FBS (Fig. 1E). As EVs were derived from normoxic donor cells that are virtually devoid in LDs (see Fig. 1D, top), it was unlikely that EVs harbored significant amounts of LDs for direct transfer. Nevertheless, to exclude this possibility, hypoxic cells were exposed to EVs for 3 hours to allow internalization, and then directly stained for LDs. Under these conditions, recipient cells were LD negative (Supplementary Fig. S1C). Moreover, we found that EV-mediated LD loading was insensitive to treatment with specific inhibitors of FASN (Fasnall and GSK2194069; Supplementary Fig. S1D and S1E). Although the fluorescence dye used to analyze LD formation (LipidTox neutral lipid) is known to fluoresce specifically in association with neutral lipids, it was important to corroborate imaging results with biochemical data. We found that EVs induced a substantial accumulation of triglycerides in hypoxic cells compared with control, whereas no effect was observed in normoxia (Fig. 1F). Together, these data suggest that EVs can be processed to fuel the LD† phenotype specifically in hypoxic cells through a mechanism that is independent on intact FASN.

**Exosomes Induce Lipid Droplet Formation in Tumor Hypoxia**

Previous studies have demonstrated hypoxic induction of EV secretion, and that the composition of EVs mimics the hypoxic response of producing cells (19, 24, 25, 41). EVs may exert functional effects by direct ligand activation of cell surface receptors or through mechanisms that require EV internalization and cargo transfer. We next set out to investigate how cellular hypoxia may regulate EV binding and entry into target cells to trigger the LD† phenotype. An optimized protocol for EV fluorophore labeling designed to minimize nonspecific dye transfer to target cells was employed (29). Remarkably, we found that EV uptake was increased by approximately 2-fold in hypoxia as compared with normoxic cells at all concentrations tested, as shown by flow cytometry (Fig. 2A). Hypoxic induction of EV uptake was observed from the shortest hypoxic timepoint tested (2 hours), and at which HIF stabilization has been described previously (42), and was persistent for up to 6 hours of hypoxia. Hypoxic induction appeared transient, as we found comparable EV uptake levels in hypoxia and normoxia at 20 hours (Fig. 2B). Increased EV uptake in hypoxia was not restricted to U87 MG cells, as we found an approximately 2-fold increase in mouse HGG cells, GL261, also (Fig. 2C; ref. 26). As in U87 MG, the hypoxic effect in GL261 cells applied to different EV concentrations (Supplementary Fig. S2A), and was acute and transient, showing approximately 2-fold induction at 2 and 6 hours of hypoxia, and approximately 1.3-fold induction at 20 hours (Supplementary Fig. S2B). Moreover, human primary glioblastoma cells (U3034) grown in serum-free, stem cell permissive conditions showed an approximately 1.3-fold and significant induction of EV uptake at hypoxia as compared with normoxia (Fig. 2C). Flow cytometry data were further supported by high-resolution Airyscan confocal microscopy, demonstrating that EVs were internalized into intracellular vesicles that appeared more intense in hypoxic versus normoxic U87 MG, GL261, as well as U3034 cells (Fig. 2D). Intriguingly, however, we observed no hypoxic effect on EV cell surface binding, in either HGG cell lines or primary cells (Fig. 2E). Together, these data suggest that EV-mediated induction of the LD† phenotype may be mechanistically associated with hypoxic regulation of EV internalization. EVs may bind to and enter cells through multiple pathways, but the exact uptake mechanism is poorly understood (15, 22, 23). Proposed processes for EV uptake include direct membrane fusion and a variety of endocytic pathways, depending on the source of EVs and experimental conditions (43–45). The above findings show that EVs display concentration-dependent uptake kinetics at hypoxia and normoxia (Fig. 2A; Supplementary Fig. S2A), and incubation at 4°C efficiently attenuated uptake (Supplementary Fig. S2C). Further visualization
using high-resolution Airyscan live-cell confocal microscopy revealed that internalized EVs appear as mobile vesicles in the cytoplasm (Supplementary Video S1). These data are consistent with hypoxic regulation of an endocytic EV uptake mechanism, rather than direct membrane fusion. Hypoxic remodeling of endocytosis may occur through two major mechanisms, either independent or dependent on HIF1α and 2α (HIF1/2α), which are key sensors of cellular oxygen availability (46–49). HIFs have been shown to alter the expression of, for example, Rab proteins and endocytic coat proteins, and to induce lipid uptake through increased expression of lipoprotein receptors (VLDLR, LRP1, and SR-B1) and lipid transporting proteins (CD36 and FABP4; refs. 6, 8, 50, 51). Our finding that hypoxic induction of EV uptake was transient (Fig. 2B; Supplementary Fig. S2B) is consistent with an HIF-dependent response, which in many cases appear as transient over a period of 24–48 hours. We thus, next explored the potential role of HIFs in hypoxic regulation of EV uptake by applying different strategies to inhibit their function (29). We next explored the role of HSPGs in hypoxic induction of EV uptake by applying various macromolecular ligands (54) and have been implicated as one of the main pathways for endocytic EV uptake (29). We included competitive inhibition of HSPG by the HS mimetic heparin (Fig. 4B), digestion of cell surface HSPG by specific HS lyases (Fig. 4C), pharmacologic inhibition of HSPG biosynthesis by the false substrate, 4-nitrophenyl β-D-xlyopyranoside (PNP-xyl; Fig. 4D), as well as inhibition of HS sulfation and polyanionic charge by NaClO3 treatment (Fig. 4E). EV internalization was visualized by confocal microscopy and quantified by flow cytometry analysis. Interestingly, regardless of strategy used to interfere with HSPG function, we consistently found inhibition of hypoxia-induced EV uptake.

**Hypoxic induction of EV uptake through increased HSPG receptor endocytosis**

HSPGs are defined by the conjugation with highly polyanionic HS polysaccharide chains that serve as cell surface attachment sites for various macromolecular ligands (54) and have been implicated as one of the main pathways for endocytic EV uptake (29). We next explored the role of HSPGs in hypoxic induction of EV uptake by applying different strategies to inhibit their function (Fig. 4A). These strategies included competitive inhibition of HSPG by the HS mimetic heparin (Fig. 4B), digestion of cell surface HSPG by specific HS lyases (Fig. 4C), pharmacologic inhibition of HSPG biosynthesis by the false substrate, 4-nitrophenyl β-D-xlyopyranoside (PNP-xyl; Fig. 4D), as well as inhibition of HS sulfation and polyanionic charge by NaClO3 treatment (Fig. 4E). EV internalization was visualized by confocal microscopy and quantified by flow cytometry analysis. Interestingly, regardless of strategy used to interfere with HSPG function, we consistently found inhibition of hypoxia-induced EV uptake.
Statistically significant inhibition of EV uptake was also observed in normoxic cells, indicating that the HSPG dependence is not exclusive to hypoxia. While heparin and chlorate treatment completely abrogated hypoxic induction of EV uptake, a small, but significant hypoxic effect remained with PNP-xyl and HS lyase treatment, possibly due to the incomplete eradication of HSPG by these treatments.

HSPGs may operate as bona fide endocytic receptors or as part of a larger receptor complex with, for example, lipoprotein and virus receptors (55–57). To explore whether hypoxia directly regulates HSPG receptor expression and uptake activity, we took advantage of scFv antibodies (α-HS) that were selected for specific HS epitopes by phage display (Table 1; refs. 58, 59). Anti-HS specificity for sulfated HS was validated by decreased staining of chlorate-treated as compared with untreated cells (Supplementary Fig. S3A). We could show that HSPG receptor expression was not regulated by hypoxia in U87 MG, as well as as GL261 cells (~1.4- and 1.8-fold, respectively; Fig. 5B). Flow cytometry data were supported by confocal microscopy, showing increased α-HS fluorescence in intracellular vesicles of hypoxic as compared with normoxic cells (Fig. 5C). To test whether this effect was HS epitope specific, we used another α-HS (AO4B08), showing similar results (Supplementary Fig. S3B). The hypoxic effect was specific for HS, as CS, which is structurally similar to HS, consistently showed similar cell surface expression and internalization at normoxic and hypoxic conditions, using α-CS antibodies with different epitope specificity (Fig. 5D; Supplementary Fig. S3C). Together, these data suggest hypoxic regulation of HSPG receptor internalization as an underlying mechanism of increased EV uptake at hypoxic conditions.

**Increased lipid raft-mediated EV internalization in hypoxia**

We next explored how increased HSPG-mediated EV uptake may be mechanistically linked to hypoxic regulation of a specific endocytic route. Endocytosis of HSPG receptor ligands has been shown to follow a variety of pathways, including classical clathrin-associated and membrane raft-mediated endocytosis, and macrophagocytosis (57, 60, 61). We found that both in normoxia and hypoxia,
Figure 3.
Hypoxic induction of EV endocytosis does not depend on HIF transcriptional regulation. A and B, KD of HIF1α and HIF2α (left). Normoxic (N) and hypoxic (H) U87 MG cells were analyzed for HIF1α (A) and HIF2α (B) by Western blotting, with tubulin as loading control. siCtrl, transfection with a nontargeting siRNA; siHIF1α and siHIF2α, transfection with two different siRNAs (#1 and #2) specific for the respective HIF. Shown are representative immunoblots from three independent experiments. Flow cytometry analysis of EV uptake at the conditions described above shows no role of HIFs in EV uptake (n = 3, from one representative experiment; right). C, Pharmacologic HIF stabilization in normoxic cells by desferrioxamine (DFO) or dimethyloxalylglycine (DMOG) does not induce EV internalization (n = 3, from one representative experiment).

A–C, Flow cytometry data were normalized to normoxic control (ctrl) and are presented as the mean fold ± SD. NS, nonsignificant.

Figure 4.
Hypoxic induction of EV uptake depends on cell surface HSPGs. A, Schematic outline of approaches used to inhibit HSPG receptor function. B, EV uptake in normoxic (N) and hypoxic (H) U87 MG (top) and GL261 (bottom) cells in the absence or presence of heparin, as visualized by confocal microscopy (left), and quantified by flow cytometry (right; n = 6, from two independent experiments). C, EV uptake in normoxic and hypoxic U87 MG cells without (control, ctrl) or with pretreatment with HS lyases to eradicate cell surface HSPG (n = 9, from three independent experiments). D, EV uptake in normoxic and hypoxic U87 MG cells pretreated with vehicle (DMSO; control) or PNP-xyl to inhibit HSPG biosynthesis (n = 9, from three independent experiments). E, EV uptake was assessed by confocal microscopy (left) and flow cytometry (right) with no treatment (control) or treatment with NaClO₃ to inhibit HSPG sulfation and ligand binding (n = 6, from two independent experiments). B–E, Flow cytometry data were normalized to normoxic control (set at 1) and are presented as the mean fold ± SD (*, P < 0.05; NS, nonsignificant).
internalized EVs show no apparent colocalization with transferrin, that is, a ligand and marker of clathrin-mediated endocytosis, and limited colocalization with the macropinocytosis marker, dextran (Fig. 6A). Interestingly, there was a clear vesicular codistribution of internalized EVs and the membrane raft marker, CtxB, in hypoxic cells, indicating that EVs were diverted into this pathway (Fig. 6A). EV and CtxB colocalization was visualized by high-resolution Airyscan live-cell confocal microscopy, showing mobile intracellular vesicles positive for both ligands (Supplementary Video S2). The above observations were supported by increased CtxB uptake in hypoxic as compared with normoxic cells, whereas dextran and transferrin uptake was not increased (Fig. 6B). Hypoxic induction of CtxB uptake was consistent in U87 MG and GL261 cells (~1.3-fold up by hypoxia in both cell lines; Fig. 6B, right). We employed the drug MCD to preferentially inhibit membrane raft–mediated endocytosis through disruption of cholesterol-rich membrane microdomains (62). In glioma cells, MCD treatment efficiently abrogated plasma membrane cholesterol density, as determined by filipin III staining (Supplementary Fig. S4A) and, accordingly, CtxB internalization was substantially decreased (Supplementary Fig. S4B). We found statistically significant inhibition of EV uptake by MCD treatment in normoxia and hypoxia, as visualized by confocal microscopy (Fig. 6C, left) and quantified by flow cytometry in U87 MG and GL261 cells (Fig. 6C, middle and right, respectively). Next, we explored the possibility that preloading with cholesterol, that is, an important structural component of lipid rafts, could mimic the hypoxic effect by stimulating raft-mediated endocytosis. In line with this idea, confocal microscopy showed increased lipid raft staining using surface CtxB staining and cross-linking in cholesterol-loaded cells (Fig. 6D, left), and flow cytometry concomitantly showed an approximately 2-fold increase of EV uptake in cholesterol-loaded as compared with control cells (Fig. 6D, right). We could link the above findings to the HSPG receptor pathway, as α-HS uptake was also decreased by MCD treatment (Supplementary Fig. S4C). More importantly, high-resolution imaging of hypoxic cells showed colocalization of the major cell surface HSPG, syndecan-1 (SDC1; refs. 56, 61), and EVs to endocytic vesicles that were positive for CtxB (Fig. 6E, left). Moreover, MCD treatment efficiently inhibited their intracellular codistribution (Fig. 6E, right). Together, these data identify a specific role of the membrane raft pathway in hypoxia-induced endocytosis of EVs.

**EV lipid transfer and induction of the LD^{+} phenotype through the HSPG endocytic pathway**

The above data indicate that HSPGs are required for hypoxia-induced EV uptake. However, under hypoxic conditions, approximately 15% to 45% uptake activity remained in cells with deficient HSPG function as compared with controls (Fig. 4). Thus, we next investigated the functional relevance of HSPGs in EV-induced LD loading. Interestingly, EV-dependent LD formation was attenuated by heparin treatment in hypoxic cells (Fig. 7A). Also, pretreatment with HS lyses to eradicates cell surface HSPGs (see Supplementary Fig. S5A for validation) significantly reversed LD induction by EVs, as shown by confocal imaging and subsequent LD area quantification (Fig. 7B).

The above data suggest that LD induction by EVs was not due to direct transfer of LĐs carried by EVs, and did not require intact de novo lipid synthesis through FASN (Supplementary Fig. S1). To explore whether EV-incorporated lipid could be directly processed into LD storage, we next employed a fluorescence fatty acid analogue (BODIPY-FA) equivalent to a 18-carbon and known to be readily metabolized by live cells into triglycerides and other natural lipids (36–38). First, we could show that BODIPY-FA was efficiently incorporated into intracellular vesicle membranes of U87 MG cells (Supplementary Fig. S5B), and that EVs isolated from these cells (BODIPY-FA-EVs) had incorporated the probe, and were taken up similarly to PKH-labeled EVs by recipient unlabeled cells, as assessed by flow cytometry and confocal microscopy (Supplementary Fig. S5C). Using high-resolution imaging, we found that BODIPY-FA-EVs can transfer the fatty acid probe for

### Table 1. Epitope specificities of CS and HS antibodies used for assessing cell surface PG and PG internalization.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>HS/CS Epitope</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>CS-56</td>
<td>CS, CS-A, CS-D</td>
<td>(32)</td>
</tr>
<tr>
<td>GD3Gp</td>
<td>CS, CS-E</td>
<td>(35)</td>
</tr>
<tr>
<td>AO4B08</td>
<td>HS-I, IdoA, NS</td>
<td>(34)</td>
</tr>
<tr>
<td>HS4C3</td>
<td>HS, 20S, 60S</td>
<td>(33)</td>
</tr>
</tbody>
</table>

*Abbreviations: 2OS, 2-O-sulfated; 3OS, 3-O-sulfated; 6OS, 6-O-sulfated; CS-A, GlcA-GalNAc; CS-D, GlcA,2S-GalNAc,6S; CS-E, GlcA-GalNAc,4S; 6S, IdoA, iduronic acid; NS, N-sulfated.*

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**Figure 5.** HSPG internalization is induced by hypoxia (H). A, Anti-HS antibody (α-HS) binding to normoxic (N) and hypoxic U87 MG and GL261 cells at 4°C (n = 3). B, α-HS uptake in normoxic and hypoxic U87 MG and GL261 cells (n = 9, from three independent experiments). C, CtxB uptake in normoxic and hypoxic U87 MG and GL261 cells (n = 9, from three independent experiments). A, B, and C, Results were normalized to normoxia (set at 1) and are presented as the mean fold ± SD (*, P < 0.05; NS, nonsignificant).
storage into LDs, as suggested by strong colocalization between BODIPY-FA per se not directly incorporated into preestablished LDs (Supplementary Fig. 5D). Together, these data suggest that EV-dependent LD induction involves direct transfer of EV-associated lipid cargo. Finally, we could show that the increased lipid stores observed in hypoxic cells following EV uptake were efficiently consumed upon reoxygenation (Fig. 7D; Supplementary Video S3), suggesting that EV-induced LDs were available to enter glioma cell metabolism under conditions of restored mitochondrial respiration. Together, our results suggest that EVs fuel the LD⁺ phenotype mainly through direct lipid transfer via a hypoxia-sensitive, HSPG-dependent endocytic route (Fig. 7E).

**Discussion**

The importance of enhanced utilization of exogenous nutrient sources in malignant tumors is gaining increased interest (6, 40, 63–65). In this context, the role of endocytosis as a feeding mechanism of extracellular macromolecules provides a new perspective in cancer cell metabolic adaptation. Here, we provide evidence that EVs can fuel hypoxic glioma cells to acquire the LD⁺ phenotype. We show that this effect is associated with increased EV internalization through a hypoxia-sensitive mechanism dependent on HSPG receptor and lipid raft–mediated endocytosis. Importantly, various strategies to interfere with HSPG function were sufficient to attenuate LD loading, indicating that the HSPG uptake route is relevant for intracellular EV processing into LDs. 

De novo lipid biosynthesis is energy demanding, and glioma cells residing in the hypoxic niche have a craving for extracellular lipids.
from local sources (6, 13). In this context, EVs from the tumoral interstitium constitute an attractive source of metabolites that can be retrieved by endocytosis. We show that EVs induce the LD<sup>+</sup> phenotype independently of intact FASN activity, and that EV-associated lipid cargo can be incorporated into LDs for storage in glioma cells when residing in a hypoxic environment. Dysfunctional mitochondrial respiration in hypoxia leads to impaired tricarboxylic acid cycle and inhibition of fatty acid oxidation. Thus, in the context of EV-driven LD accumulation, it is also important to consider hypoxic impairment of lipid catabolism. Accordingly, the increased lipid stores observed in hypoxic cells following EV uptake were efficiently consumed upon reoxygenation. Thus, in our model, EV-driven LD accumulation in hypoxic cells is likely to derive from a synergy between increased EV internalization and cargo transfer through HSPG, and impaired mitochondrial lipid oxidation.

The signaling and metabolic status of cancer cells have been intimately associated with modulation of endocytic traffic of specific receptor proteins, for example, increased import of extracellular proteins was found in cancer cells harboring oncogenic RAS mutations (64). Of relevance in the context of this study, the cell surface HSPG SDC1 (or CD138) was upregulated by KRAS to drive nutrient salvage in pancreatic adenocarcinoma cells (60). HSPGs are proteins decorated with polyanionic glycosaminoglycans found in the extracellular matrix and at the cell surface, where they can act as autonomic internalizing receptors or be part of a multi-receptor complex in partnership with, for example, viral and lipoprotein receptors (57, 66). In hepatocytes, the HSPG can function as an independent endocytic receptor. HSPG-mediated endocytosis of EVs depends on intact lipid rafts, leading to the transfer of EV-associated lipid to the LD compartment of glioma cells. LD induction by EVs depends on hypoxia, which was shown to induce HSPG internalization and HSPG-mediated EV uptake. LDs were consumed upon reoxygenation, suggesting that impaired lipolysis and mitochondrial oxidative capacity contribute to EV-induced LD storage.

Figure 7. EV induction of the LD<sup>+</sup> phenotype depends on HSPG. A, Confocal microscopy visualization of LDs in normoxic (N) and hypoxic (H) U87 MG cells incubated in serum-free medium (control, ctrl), or in medium with EVs in the absence (EV) or presence of heparin (EV + hep; left), LD area quantification from corresponding confocal images. Data were normalized for normoxic control and are expressed as the mean fold ± SEM (n ≥ 3 images, from two independent experiments). B, Decreased EV-induced LD formation by HS lyase treatment. Representative confocal images and corresponding image quantification as in A. A and B, Data were normalized for normoxic control and are expressed as the mean fold ± SEM (n ≥ 3 images, from two independent experiments). C, Transfer of EV-associated lipid to LDs. Hypoxic U87 MG cells were stained for LDs with LipidTox (red) following 48-hour incubation with EVs isolated from BODIPY-FA–labeled donor cells (yellow), either in the absence (EV) or presence of heparin (EV + hep). Orange in merged image indicates colocalization of EV-associated BODIPY-FA with LDs. D, Consumption of EV-induced LDs. Hypoxic U87 MG cells were incubated for 48 hours in serum-free medium (control) or with EVs and stained for LDs directly or following 24 or 48 hours of reoxygenation (reox). C and D, Shown are representative images from two independent experiments. Scale bars = 20 μm. E, Schematic representation of how scavenging of EVs can fuel the LD phenotype in glioma cells. HSPG-mediated endocytosis of EVs depends on intact lipid rafts, leading to the transfer of EV-associated lipid to the LD compartment of glioma cells. LD induction by EVs depends on hypoxia, which was shown to induce HSPG internalization and HSPG-mediated EV uptake. LDs were consumed upon reoxygenation, suggesting that impaired lipolysis and mitochondrial oxidative capacity contribute to EV-induced LD storage.
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receptor for triglyceride-rich lipoproteins (56, 67). Previous studies have suggested that glioma cells take up significantly increased levels of extracellular lipoprotein as compared with normal astrocytes, and that this occurs through EGFR-mediated induction of lipoprotein receptor activity (68). Other studies suggested that hypoxia-mediated enhancement of lipoprotein uptake requires initial attachment to HSPG, as well as hypoxic induction of very LDL receptor, that is, a known HIF-responsive protein (40, 50, 69). In this study, we, for the first time showed that, increased import of EVs is directly associated with hypoxic regulation of HSPG membrane raft endocytosis. Thus, while there is a differential dependence on HIF-regulated endocytosis, hypoxic induction of lipoprotein and EV uptake shares the requirement of HSPG receptors. It may be proposed that hypoxia regulates endocytic traffic through HIF-mediated transcriptional regulation, as well as HIF-1-independent mechanisms, that together have broad impact on nutrient supply and the metabolic status of glioma cells. To what extent modulation of HSPG expression, structure, and membrane transport kinetics in vivo exert control over EV and lipoprotein bioavailability and metabolic adaptation during hypoxic conditions warrants further investigation.

While abnormal glucose uptake via glucose transporters and aerobic glycolysis (Warburg effect) for long has been recognized as hallmarks of cancer, recent studies identify altered lipid metabolism and import as prominent features of cancer cell adaptation in the hypoxic tumor microenvironment (5, 70–73). However, lipids not only offer an efficient energy source and function as signaling molecules, but also form the structural basis of biological membranes. Currently, we can only speculate about how increased EV uptake and LD accumulation may participate in hypoxic paracrine signaling by increased secretion of key effector molecules (e.g., VEGF and HB-EGF), involved in shaping the proangiogenic and immunosuppressive HGG microenvironment (72). Studies in experimental models suggest that targeting of lipid metabolism efficiently interferes with tumor progression and metastasis, and different strategies to inhibit cholesterol and fatty acid biosynthesis are currently explored in the clinical setting (7, 10, 13, 68, 74, 75). However, based on our findings and previous studies, it may be anticipated that hypoxic cancer cells subjected to treatment with lipid biosynthesis inhibitors are rescued by compensatory salvage of systemic and local EV sources through increased endocytosis.

In summary, we have found an association between increased EV internalization and the LD+ malignant phenotype, which has the potential to serve for improved adaptation in the hypoxic tumor microenvironment. This motivates further studies aimed at establishing therapeutic strategies targeted at the endocytic machinery that should be combined with available inhibitors of lipid biosynthesis.

**Authors’ Disclosures**

No disclosures were reported.

**Authors’ Contributions**

M. Cerezo-Maga: Conceptualization, data curation, formal analysis, validation, methodology, writing—original draft, writing—review and editing. H.C. Christianson: Data curation, formal analysis, methodology, writing—review and editing. T.H. van Kuppevelt: Resources, methodology, writing—review and editing. K. Forsberg-Nilsson: Resources, validation, methodology, writing—review and editing. M. Belting: Conceptualization, supervision, funding acquisition, validation, writing—original draft, project administration, writing—review and editing.

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**References**

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