Neurochemical Research (2019) 44:1113–1126
https://doi.org/10.1007/s11064-019-02742-3

ORIGINAL PAPER

Resveratrol Abrogates Hypoxia-Induced Up-Regulation of Exosomal Amyloid-β Partially by Inhibiting CD147

Junchao Xie[1] · Xiaoyan Li[1] · Yuchen Zhou[1] · Jiayan Wu[1] · Yan Tan[1] · Xiaoye Ma[1] · Yichen Zhao[1] · Xueyuan Liu[1] · Yanxin Zhao[1]

Received: 12 November 2018 / Revised: 23 January 2019 / Accepted: 24 January 2019 / Published online: 15 February 2019

Abstract

Hypoxia promotes both total extracellular and exosomal amyloid-β (Aβ) production and aggravates Alzheimer's disease (AD). Resveratrol (RSV) has been proved to be neuroprotective in AD models, and down-regulated the expression of CD147, an additional subunit of γ-secretase. In this study, we aimed to explore the role and mechanisms of RSV in hypoxia-induced upregulation of Aβ, especially exosomal Aβ. SH-SY5Y cells and HEK293 cells overexpressing amyloid precursor protein (APP) as well as C57BL/6 mice were treated with RSV and exposed to hypoxic conditions. The expression of SIRT1 or CD147 was modulated by transfection of specific siRNAs or plasmid. Aβ1-40 and Aβ1-42 levels were determined by ELISA. Hypoxia increased the levels of both Aβ1-40 and Aβ1-42 in the hippocampal lysates and serum-derived exosomes of mice. Hypoxia also increased both Aβ1-40 and Aβ1-42 levels in the total culture medium (CM), cell-derived exosomal lysates, and exosome-free CM of both cell lines. Treatment with RSV abrogated these changes in Aβ expression, inhibited the hypoxia-induced down-regulation of SIRT1 and up-regulation of CD147. Knockdown of SIRT1 promote total Aβ level but has no effect on exosomal Aβs expression. Knockdown of CD147 inhibits both total and exosomal Aβs expression. Furthermore, overexpressing CD147 in cells exposed to hypoxia facilitated the production of Aβ1-40 and Aβ1-42, while application of RSV reduced the CD147 expression as well as Aβ levels in both exosomes and exosome-free CM. These results suggested that RSV abrogated hypoxia-induced up-regulation of total and exosomal Aβ partially by inhibiting CD147.

Keywords Amyloid-β · Exosome · Hypoxia · CD147 · Resveratrol

Introduction

Alzheimer's disease (AD) is a major age-related neurodegenerative disorder characterized by amyloid deposits derived from amyloid β-peptide (Aβ) [1]. Aβ is produced from the proteolysis of amyloid precursor protein (APP) after its sequential cleavage by β-secretase and γ-secretase [2]. In the extracellular microenvironment, Aβ not only exists as oligomers in the extracellular matrix, but also in the extracellular vesicles, including exosomes [3]. These exosomes are a type of bilayer extracellular microvesicles with 30–150 nm diameter and functions as delivery packets secreted by cells [4]. Exosomes may play a role in the transport of toxic Aβ and APP-(C-terminal fragment) CTF, further facilitating the deposit of amyloid and aggregating as plaques in the brain [5–7].

The concept of mixed dementia proposed in recent years suggests vascular pathologies in AD, leading to insufficient energy supply and hypoxia to the brain tissue [8, 9]. It has been revealed that hypoxia can increase the amyloidogenic processing of APP and the accumulation of Aβ [10–12]. In addition, serum Aβ1-40, Aβ1-42 levels in obstructive sleep apnea syndrome patients were significantly elevated and are positively correlated with

Junchao Xie and Xiaoyan Li contributed equally to this work and co-first authors.

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s11064-019-02742-3) contains supplementary material, which is available to authorized users.

✉ Xueyuan Liu
lxyshtj@163.com

✉ Yanxin Zhao
zhao_yanxin@126.com

[1] Department of Neurology, Shanghai Tenth People's Hospital, Tongji University School of Medicine, 301 Middle Yanchang Road, Shanghai 200072, China

the severity of hypoxia, suggesting an association between hypoxia and Aβ production [13]. Also, animal studies have revealed that hypoxia facilitated Aβ deposition in mice brain [12]. Therefore, the relation between hypoxia and AD pathological changes, especially the production and accumulation of Aβ, might serve as a breakthrough point to further deepen the research on the pathological mechanism of AD. Our recent study reported that hypoxia not only increases the total extracellular Aβ1-40 and Aβ1-42 level, but also amplifies the Aβ1-40 and Aβ1-42 level in exosomes derived from APP-overexpressing SH-SY5Y cells [14]. Therefore, it remains valuable to explore safe and feasible methods for the inhibition of Aβ production in exosomes.

Recent studies indicated that resveratrol (RSV), a polyphenol mainly found in grapes and red wine, plays a neuroprotective role in AD partially through activating SIRT1 [15–17]. It decreases the amyloidogenic cleavage of APP and reduces the aggregation of Aβ [18, 19]. However, the effect of RSV on Aβ in exosomes under hypoxic conditions has not been reported. Hence, in this study, we explored the role of RSV in Aβ production and the underlying mechanisms both in vivo and in vitro hypoxic model.

Materials and Methods

Animals and Hypoxia Conditioning

6–8 weeks-old specific pathogen-free (SPF) male C57BL/6 mice were purchased from Shanghai SLAC Laboratory Animal Co. Ltd (Shanghai, China) and were housed in SPF environment supplied with food and water *ad libitum*. A 12-h/12-h light/dark cycle was maintained. All animal experiments were conducted in compliance with Institutional Animal Care and Use Committee guidelines.

To create a hypoxic environment, a specially designed hermetic chamber with a air-intake hole and a vent was used. Consistent airflow (consist of 8% oxygen and 92% nitrogen) was supplied through the air-intake hole. An oxygen concentration detector (Smart Sensor AR8100, China) was used to confirm the oxygen concentration in the chamber. For hypoxic model, mice were kept in this chamber and supplied with 8% O_2 for 1, 3, and 5 h. The mice in the control group were exposed to the air (21% O_2) in the same chamber. In some of the experiments, mice were fed with diet supplemented with 150 mg/kg or 300 mg/kg RSV per day for 4 weeks and were then subjected to hypoxic conditioning.

Mice were sacrificed immediately after exposure to hypoxic conditions and the whole blood and brains were quickly collected. Hippocampus was promptly separated and collected for further experiments.

Cell Lines and Hypoxia Conditioning

Human neuroblastoma SH-SY5Y cells with stable overexpression of human wild-type APP^{695} cDNA (SH-SY5YAPP^{695}) were kindly provided by Professor Sheng-Di Chen (Department of Neurology and Institute of Neurology, Ruijin Hospital, Shanghai JiaoTong University School of Medicine, Shanghai). HEK293T cell line was from the Neuroscience Institute of Shanghai Tenth People's Hospital.

Cells were cultured with DMEM media supplemented with 10% exosome-free fetal bovine serum (FBS, Gibco). For hypoxia conditioning, cells were cultured in a hypoxia incubator (Thermo Scientific™ Forma™ Steri-Cycle™ i60 CO_2) with O_2 concentration of 2% for 6, 12, and 24 h. Control cells were kept in another incubator with mixed gases of 21% O_2, 5% CO_2, and balance N_2. In some of the experiments, RSV (25 or 50 μM), and DMSO (diluent of RSV) were applied to the cells 1 h before exposure to hypoxic conditions.

Plasmid over-expressing CD147 (GV208-CD147-flag) or APP (pEASY-Blunt M2-APP-Myc) was constructed. To create APP-overexpressing HEK293T cells (HEK293T-APP), plasmid over-expressing APP (pEASY-Blunt M2-APP-Myc) was constructed and transfected into the cells 24 h before hypoxia conditioning.

In some of the experiments, plasmid or siRNA transfection was conducted 24 h before hypoxia conditioning. Lipofectamine® 3000 (Invitrogen, Life Technologies) transfection reagent was used according to the manufacturer's instructions. The CD147 siRNA, SIRT1 siRNA and negative control siRNA were designed and synthesized by Genepharma (Shanghai, China). 20 μM siRNA resolved in RNase-free water. Right before transfection, siRNA was diluted by serum-free culture medium (CM) to a concentration of 0.8 μM. Lipo3000 reagent was also diluted with serum-free CM (3:100). The diluted siRNA and lipo3000 was then mixed and incubated at room temperature for 5 min. Thereafter, the siRNA and Liop3000 mix was added to the cells. The final concentration of siRNA in the culture plate was 40 pM. The cells were incubated for 24 h and were then exposed to hypoxia for another 24 h.

Antibodies and Reagents

The following primary antibodies were used: mouse anti-CD147 antibody (1:1000 for WB, SantaCruz Biotechnology, sc-25273), rabbit anti-CD147 antibody (1:50 for co-immunoprecipitation, Abcam, ab108317), rabbit anti-SIRT1 antibody (1:1000 for WB, Abcam, ab189494), rabbit anti-β-actin antibody (1:5000 for WB, Abcam,

ab8227), rabbit anti-ALIX antibody (1:1000 for WB, Sigma-Aldrich, SAB4200477), and rabbit anti-Flotillin 1 antibody (1:1000 for WB, Abcam, ab41927). RSV was purchased from Sigma Aldrich (product No: R5010, Sigma Aldrich).

Isolation of Exosomes

Exosomes were isolated from the whole blood of the mice or cell CM. The serum of mice was obtained by centrifuging the whole blood sample. Exosomes in the serum or CM were precipitated with ExoQuick exosome precipitation solution (EXOQ; System Biosciences, Inc., Mountain View, CA, USA). Briefly, 1/5th volume of ExoQuick reagent was added to the serum or cell CM with inhibitor cocktails, followed by refrigeration overnight. The ExoQuick/biofluid mixtures were then centrifuged at 1,500 × *g* for 30 min and the supernatants were aspirated and stored for further use (exosome-free serum or CM). The residual ExoQuick solution was then centrifuged at 1,500 × *g* for 5 min and all fluid traces were removed by aspirating tenderly to precipitate exosomes. For mice whole blood, exosomes extracted from the same volume of blood samples (1.5 ml) were subjected to the following study for comparison. For cell CM, exosomes derived from the same number of cells were collected and used for further experiments.

Transmission Electron Microscopy (TEM) Characterization of Exosomes

The extracted exosomes were fixed by 1% glutaraldehyde for 2 h at room temperature. Then the extracted exosomes were loaded on the carbon-coated electron microscopy grids and were negatively labeled with phosphotungstic acid for 5 min. The microscopic images were obtained with Phillip CM120 transmission electron microscope operated at 120 kV.

ELISA

Mice hippocampal lysates, cell CM or exosome-depleted mice serum were collected. Halt Protease Inhibitor Cocktail (Thermo Scientific, Rockford, USA) was added to the samples to prevent degradation of Aβ. The concentrations of Aβ1-40 and Aβ1-42 in the hippocampal lysates and SH-SY5YAPP^{695} cell CM were determined using mouse and human Aβ ELISA kits (both Aβ1-40 and Aβ1-42), respectively (Elabscience Biotechnology, Wuhan, China). To detect the exosomal Aβ level, exosomal pellets were re-suspended and sonicated in the diluent buffer using the ELISA kits. The resultant solutions were then added to the plate and the following procedures were performed according to the manufacturer's instructions.

Western Blot

Total protein of mice hippocampus and SH-SY5YAPP^{695} cells were extracted with RIPA buffer (Beyotime, Shanghai, China) supplemented with Halt Protease Inhibitor Cocktail (Thermo Scientific, Rockford, USA) and quantified by bicinchoninic acid assay (BCA) kit (Pierce, Rockford, IL, USA). 50 μg of proteins was loaded, with a concentration of 1 μg/μl and separated by 10% SDS–PAGE and was then transferred onto the nitrocellulose membranes (Bio-rad). The membranes were then blocked in TBS containing 5% bovine serum albumin (BSA, Sigma Aldrich) for 1 h at room temperature. The membranes were incubated in primary antibodies diluted in blocking buffer at 4 °C overnight. After rinsing three times, the membranes were incubated with IRDye800® Conjugated Mouse IgG (H&L) Antibody and IRDye700® Conjugated Rabbit IgG (H&L) Antibody (Li Cor Biosciences, NE, USA) at room temperature for 1 h. After rinsing three times, the membranes were scanned with the Odyssey infrared imaging system (Li Cor Biosciences).

Immunofluorescence Staining

Mice were sacrificed and the brains were snap-frozen in optimal cutting temperature (OCT) compound (Sakura Finetechnical, Tokyo, Japan). For immunofluorescence staining, the OCT-embedded brains were cut into 10 μm-thick sections and mounted on adhesive microscope slides (Citotest Labware Manufacturing, Jiangsu, China). The sections were fixed with ice-cold acetone for 10 min and were then blocked with 5% BSA for 60 min at room temperature. Subsequently, the sections were incubated with rabbit anti-CD147 antibody overnight at 4 °C. After being rinsed with PBS trice, the sections were further incubated with FITC-conjucated goat anti-rabbit IgG for 1 h at room temperature. After counterstained with 4,6-diamidino-2-phenylindole (DAPI), the stained sections were visualized under a confocal microscope (Carl Zeiss, LSM710, Jena, Germany). Negative controls were performed by omitting primary antibodies.

Statistical Analysis

Experiments were done at least three times. Data are expressed as mean ± SD. Comparisons were done with one-way ANOVA followed by Tukey's multiple comparison (when comparing at least three experimental groups) or two-tailed Student's *t* test(when comparing two groups). A P value of < 0.05 was considered statistically significant.

Results

Hypoxia Increased Total Aβ level and Regulated the Expression of SIRT1 and CD147 In Vitro and In Vivo

SH-SY5YAPP^{695}, HEK293T-APP cells and mice were exposed to hypoxic conditions at different time intervals. The CM of the cells and the hippocampal lysates were obtained and subjected to detect the total Aβ level by ELISA. After exposure to hypoxic conditions, the Aβ1-40 and Aβ1-42 levels in the CM were gradually increased in 24 h (Fig. 1a, b, e, f). Similarly, after the exposed to hypoxic conditions for 5 h, Aβ1-40 and Aβ1-42 levels in the hippocampal lysates of the mice were also increased gradually (Fig. 1i, j).

Previous studies indicated that CD147 may act as a regulatory subunit of the γ-secretase and in turn may regulate the production of Aβ [20], and recent studies suggested that SIRT1 is able to regulate several pathological events in AD [21]. Herein, we investigated CD147 and SIRT1 expression under hypoxic conditions in SH-SY5YAPP^{695}, HEK293T-APP cells and mice brain. WB in cell lysates and mice brain tissue revealed that hypoxia induced a significant down-regulation of SIRT1 while an up-regulation of CD147 (Fig. 1c, d, g, h, k–i).

Hypoxia Increased Exosomal Aβ level In Vitro and In Vivo

We then extracted the exosomes from the CM of SH-SY5YAPP^{695} and HEK293T cells over expressing APP, as well as from the serum of the mice. The extracted exosomes showed typical lipid bi-layer structure with a 30–150 nm-diameter (Fig. 2a, b, m) and expressed the exosome-specific markers, ALIX and Flotillin 1 (Fig. 2c, d, n). Exosome lysates were then subjected to Aβ detection. After exposure

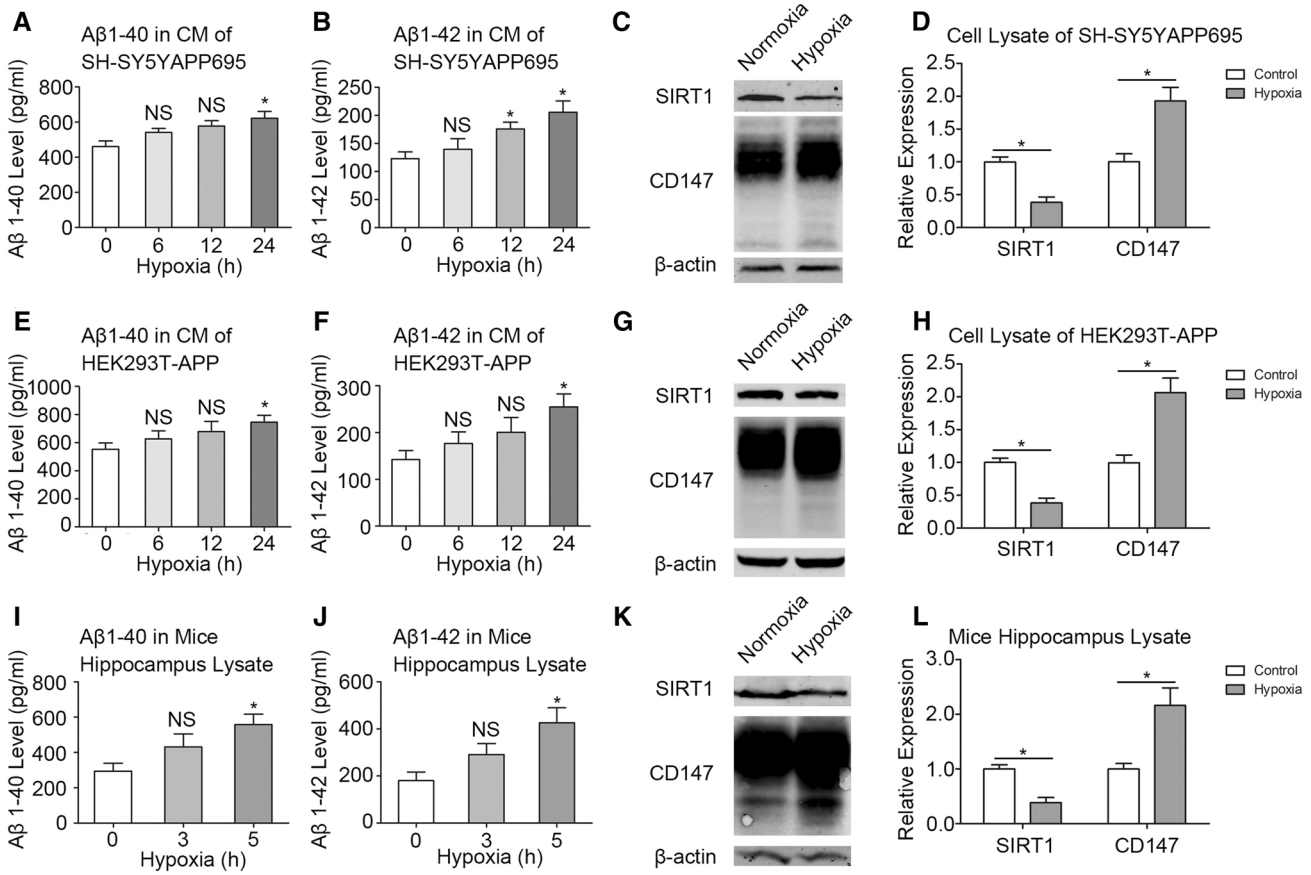


Fig. 1 Hypoxia increased total Aβ level and regulated the expression of SIRT1 and CD147 in vitro (n = 4) and in vivo (n = 6). SH-SY5YAPP^{695} and HEK293T-APP cells were exposed to hypoxia (2% O_2) for 0, 6, 12, and 24 h (**a**–**h**). C57BL/6 mice were exposed to hypoxia (8% O_2) for 1, 3, and 5 h and were sacrificed (**i**–**l**). **a**–**h** The cell CM were collected. Aβ1-40 (**a, e**) and Aβ1-42 (**b, f**) levels were measured by ELISA. The expression of SIRT1 and CD147 in cells was detected by western blot (**c, d, g, h**). I-L: Hippocampal tissues were collected and added 100 ul RIPA to the lysates. Aβ1-40 (**i**) and Aβ1-42 (**j**) levels were measured by ELISA. *represents $p < 0.05$, n = 4

to hypoxic environment, Aβ1-40 and Aβ1-42 levels were gradually increased in exosomal lysates from both cell types (Fig. 2e, f, i, j). Furthermore, in the mice serum-derived exosome lysates, Aβ1-40 was increased from 28.96 ± 4.040 to 45.70 ± 4.017 pg/ml after hypoxia exposure for 5 h (Fig. 2o). Meanwhile, the Aβ1-42 level was significantly augmented from 14.33 ± 1.219 to 25.32 ± 2.425 pg/ml (Fig. 2p).

We further detected the Aβ levels in the exosome-free CM and mice serum. After exposure of the mice to hypoxic conditions for 24 h, Aβ1-40 and Aβ1-42 levels in the exosome-free CM were increased in both SH-SY5YAPP^{695} and HEK293T-APP cells (Fig. 2g, h, k, l). Consistently, in the exosome-free mice serum, both Aβ1-40 and Aβ1-42 levels were significantly augmented after hypoxia exposure (Fig. 2q, r).

RSV Alleviated Hypoxia-Induced Aβ Production

To optimize the concentration of RSV in our in vitro study, we first treated the SH-SY5YAPP^{695} with a gradient concentration of RSV from 5 to 100 mg/L and detected the cell viability (Supplementary Fig. 1). It was found that a concentration of RSV above 50 μM (75 μM or 100 μM) leads to a decreased cell viability. Thus, we chose 25, and 50 μM in our in vitrol study. To explore the effect of RSV on hypoxia-induced Aβ production, cells were pretreated with 25 or 50 μM RSV for 1 h and exposed to hypoxic conditions for another 24 h. Results showed that RSV reduced the hypoxia induced Aβ1-40 and Aβ1-42 production in both exosomal lysates and exosome-free CM in a dose-dependent manner (Fig. 3a, h). Previous studies indicated that RSV could activate SIRT1 [17] and regulate CD147 expression in several cell types [22–24]. Here, we further detected the effect of RSV on SIRT1 and CD147 expression in our hypoxia model. SH-SY5YAPP^{695} and HEK293T-APP cells were pretreated with RSV and exposed to hypoxic conditions as described before. Pretreatment with RSV increases SIRT1 expression and reduces CD147 expression in a dose-dependent manner in both cell types (Fig. 3i, l).

We further verified the effect of RSV in vivo. We noticed that the dosage of RSV used in mice studies varied in a range of about 20–400 mg/kg (e.g. Cheang et al. [25]: 20 mg/kg; Corpas et al. [26]: 100 mg/kg; Karuppagounder et al. [27]: 300 mg/kg; Ding et al. [28]: 200 and 400 mg/kg). When it comes to prevent the production of Aβ in mice brain, a study by Karuppagounder et al. indicated that feeding mice with 300 mg/kg of RSV leads to diminished plaque formation and reduced Aβ expression [27]. Thus, we chose 150 and 300 mg/kg in our in vivo model. Mice were fed with diet supplemented with 150 mg/kg or 300 mg/kg RSV for 4 weeks and were then subjected to hypoxia for 5 h. Hippocampal tissue lysates and serum-derived exosomal lysate were then obtained from the mice and subjected to Aβ level detection. It was found that RSV reduced the hypoxia-induced Aβ1-40 and Aβ1-42 production in hippocampal tissue lysates, serum-derived exosomal lysates, as well as exosome-free serum in a dose-dependent manner (Fig. 4a, b, e–h). Similarly, RSV application rescued the hypoxia-induced reduction of SIRT1 and lowered the hypoxia-induced up-regulation of CD147 in mice brain (Fig. 4c, d). Immunofluorescence also indicated that RSV reduced the hypoxia-induced CD147 expression in mice hippocampus (Fig. 4i).

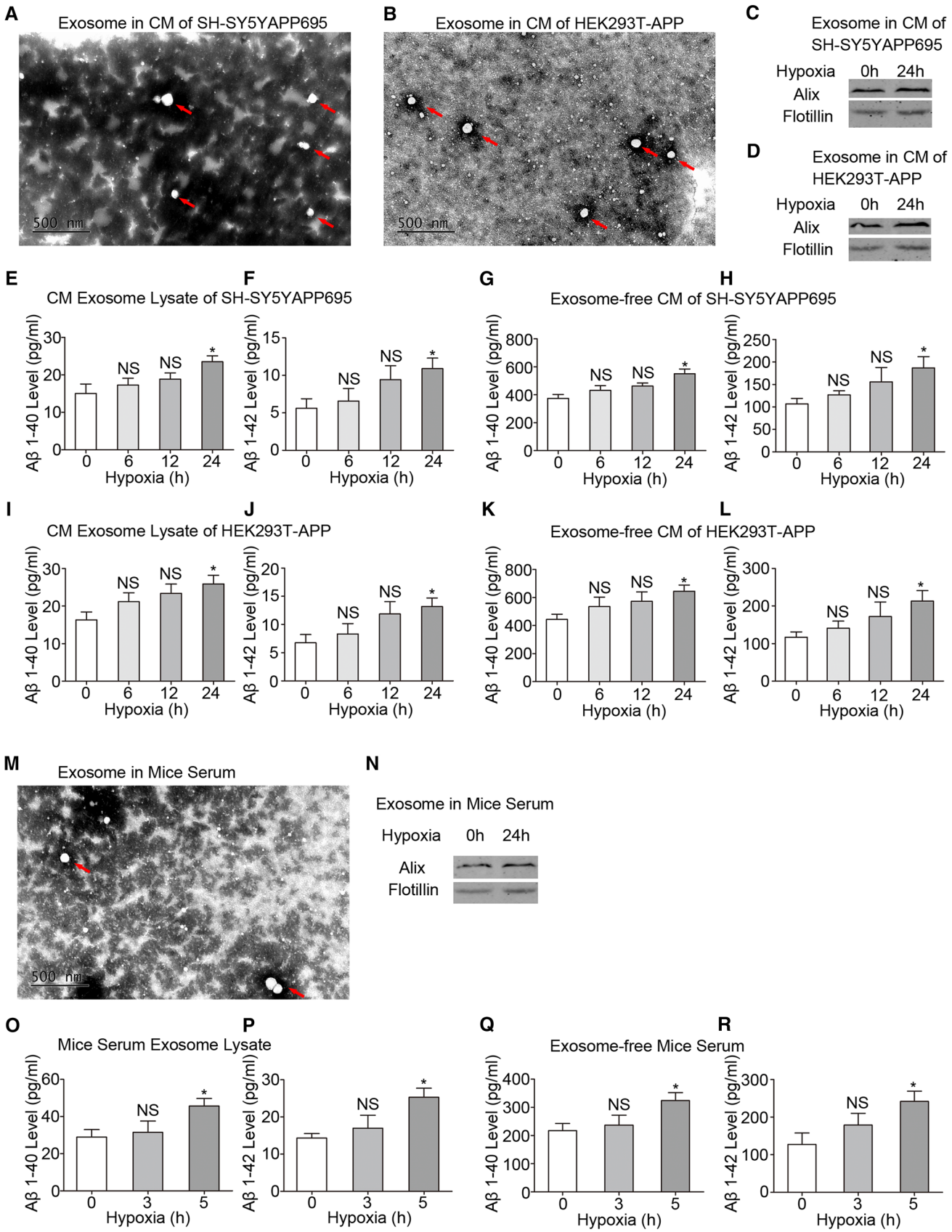
Hypoxia Induced Aβ Augmentation in Exosomes was Mediated by Increased Expression of CD147

To investigate the underlying mechanisms of hypoxia induced Aβ augmentation in exosomes, SIRT1 siRNA was synthesized and transfected into the cells 1 day before hypoxic-conditioning. After exposure to 2% oxygen for 24 h, cells were harvested. Transfection of SIRT1 siRNA successfully knocked down SIRT1 (Fig. 5a–d). Interestingly, inhibition of SIRT1 upregulated Aβ1-40 and Aβ1-42 level in exosome-depleted cell mediums but not that in exosomes (Fig. 5e–l). Besides, the expression of CD147 showed no difference after cells transfected with SIRT1 siRNA.

These results indicated that SIRT1 do not mediated the hypoxia-induced production of Aβ in exosomes. We further explored whether CD147 is the key regulator in hypoxia-induced exosomal Aβ production. CD147 siRNA was then synthesized and transfected into the cells before hypoxia exposure for 24 h. Transfection of CD147 siRNA reduced the hypoxia-induced CD147 expression by more than 40% (Fig. 6a–d). In addition, in both of the cell types, knockdown of CD147 significantly abrogated the hypoxia-induced Aβ1-40 and Aβ1-42 production in both exosomes and exosome-depleted cell supernatant (Fig. 6e–l). Knockdown of CD147 has no effect on the expression of SIRT1 (Fig. 6a–d).

Overexpression of CD147 Directly Augment Exosomal Aβ Level, and can be Inhibited by RSV Treatment

To further clarify the hypoxia-induced CD147 over-expression could regulate to the upregulation of exosomal Aβ level, we overexpressed CD147 in SH-SY5YAPP^{695} and HEK293T-APP cells (Fig. 7a, b), and observed the effect of RSV on these cells. It was found that overexpression of CD147 led to a significant upregulation of Aβ1-40 and Aβ1-42 levels in both exosomal lysate as well as exosome-free CM, while treatment with 50 μM RSV significantly abrogated these phenotypes (Fig. 7c–f).


A
Exosome in CM of SH-SY5YAPP695
500 nm
B
Exosome in CM of HEK293T-APP
500 nm
C
Exosome in CM of SH-SY5YAPP695
Hypoxia 0h 24h
Alix
Flotillin
D
Exosome in CM of HEK293T-APP
Hypoxia 0h 24h
Alix
Flotillin
E
CM Exosome Lysate of SH-SY5YAPP695
Aβ 1-40 Level (pg/ml)
Hypoxia (h)
F
Aβ 1-42 Level (pg/ml)
Hypoxia (h)
G
Exosome-free CM of SH-SY5YAPP695
Aβ 1-40 Level (pg/ml)
Hypoxia (h)
H
Aβ 1-42 Level (pg/ml)
Hypoxia (h)
I
CM Exosome Lysate of HEK293T-APP
Aβ 1-40 Level (pg/ml)
Hypoxia (h)
J
Aβ 1-42 Level (pg/ml)
Hypoxia (h)
K
Exosome-free CM of HEK293T-APP
Aβ 1-40 Level (pg/ml)
Hypoxia (h)
L
Aβ 1-42 Level (pg/ml)
Hypoxia (h)
M
Exosome in Mice Serum
500 nm
N
Exosome in Mice Serum
Hypoxia 0h 24h
Alix
Flotillin
O
Mice Serum Exosome Lysate
Aβ 1-40 Level (pg/ml)
Hypoxia (h)
P
Aβ 1-42 Level (pg/ml)
Hypoxia (h)
Q
Exosome-free Mice Serum
Aβ 1-40 Level (pg/ml)
Hypoxia (h)
R
Aβ 1-42 Level (pg/ml)
Hypoxia (h)
NS
*

◂**Fig. 2** Hypoxia increased exosomal Aβ level in vitro (n = 4) and in vivo (n = 6). **a–l** Total CM-derived from the same amount of cells were collected. Exosomes were extracted. TEM was used to observe the exosomes, which remained spherical vesicles surrounded by a bilayer lipid membrane with a diameter of 30 to 150 nm (**a, b**). Exosome-specific markers ALIX and Flotillin 1 were detected by western blot (**c, d**). Mice serum was obtained and the exosomes were extracted. Characterization of the appearance and specific markers of exosomes was performed by TEM (**m**) and western blot (**n**). **e, f, i, j, o, p**: Exosomes extracted from CM (**e, f, i, j**) and mice serum (**o, p**) were re-suspended with 100 ul diluent buffer and were sonicated. Aβ1-40 (**e, i**) and Aβ1-42 (**f, j**) levels were measured by ELISA. g, **h, k, l, q, r** Exosome-free CM (**g, h, k, l**) and serum (**q, r**) were collected during the exosome-extraction procedure. Aβ1-40 (**g, k, q**) and Aβ1–42 (**h, l, r**) level was measured by ELISA.*represents $p < 0.05$

Discussion

AD affected 10 millions of people around the world and raised huge burden on the society [29]. However, there has no definitive intervention that can effectively relieve the symptoms and delay the progression of AD. Extracellular Aβ causes toxic alteration of cell membranes. Neurofibrillary tangles consisted of phosphorylated tau and amyloid plaques containing Aβ peptides, and their accumulation lead to the destruction of synapses [30]. Aβ not only accumulates in the extracellular spaces, but also exists in the exosomes. Rajendran and Kokubo et al. found that exosomal markers such as Alix and Flotillin-1 have found to accumulate in plaques in the post-mortem brains of AD patients as well as brains of AD mice models [3, 5]. These results indicated that exosomes can act as a transporter for Aβ to move from periphery to the brain, and finally contributed to the deposit of amyloid and aggregation into plaques in the brain [5–7]. Previous studies indicated that hypoxia increased the production of Aβ in different cell types [10, 11]. Accordingly, our previous study found that after hypoxic exposure, the production of both Aβ1-40 and Aβ1-42 were enhanced in the hippocampus of C57BL/6 mice and SH-SY5YAPP^{695} cells [14]. In this current study, we observed that hypoxia increased the exosomal-derived Aβ1-40 and Aβ1-42 levels both in vitro and in vivo, which could be abrogated by the application of RSV, a polyphenol mainly found in grapes and red wine. RSV is an innoxious natural agent and has potent anti-oxidation, anti-tumor and scavenging free radical activities. Previous studies also indicated that RSV plays a neuroprotective role in AD [15, 16] by decreasing the amyloidogenic cleavage of APP and reducing Aβ aggregation [19, 22]. In our study, we confirmed that RSV not only inhibited hypoxia-induced up-regulation of total Aβ, but also abrogated the hypoxia-induced Aβ accumulation in exosomes.

RSV extends its neuroprotective function by activating SIRT1 [17, 31]. Therefore, it was expected SIRT1 might be efficient in regulating Aβ in the extracellular matrix, or even in exosomes. However, we did not determined any statistical change in exosomal-derived Aβ levels when SIRT1 was inhibited in SH-SY5YAPP^{695} or HEK293T-APP cells, although the suppressed expression of SIRT1 exacerbated the hypoxia-induced Aβ production in exosome-depleted CM. These results indicated that the hypoxia-induced production of Aβ in exosomes might not simply imputed to SIRT1 pathway, but partially due to the excessive expression of CD147, a regulator in exosomal Aβ production as we reported previously [14].

CD147 is a transmembrane protein consisting of 269 amino acids (aa) and is considered as an integral part of γ-secretase complex modulating Aβ accumulation [20]. CD147 was upregulated in neurons, axons and capillaries in the frontal cortex and thalamus of the AD brain [32].Our results indicated that hypoxia promoted both Aβ1-40 and Aβ1-42 production through inducing CD147 expression both in vitro and in vivo. Furthermore, on one hand, CD147 was validated in exosomes [33] that are derived from endosomes. On the other hand, CD147 entered the endosomes through internalization from the cell membrane. Thus, it is possible that CD147 might regulate Aβ production in exosomes by controlling Aβ packaging and cycling in the endosomes-exosomes pathway. Here, we found that hypoxia-induced Aβ accumulation in exosomes is mediated by CD147. However, the detailed mechanisms need to be addressed in the future.

Results from two studies seemed inconsistent with our current findings. Vetrivel et al. found that depletion of CD147 in cells led to increased extracellular Aβ levels, while overexpressing CD147 accelerated the degradation of Aβ by increasing the expresion of Matrix metalloproteinases (MPPs) [34]. However, their conclusions are reached in normoxia condition. Indeed, under hypoxia or ischemia condition, the distribution, expression level, and function of MMPs might be altered dramatically [35]. For example, Ashok et al. found that in chronic cerebral hypoperfusion mice model, MMP9 impaired Aβ clearance which results in an increased Aβ level [36].

Besides, Zhou et al.'s results also seemed inconsistent with ours. They found that overexpression of CD147 alone showed no significant effect on Aβ production. It indicates that CD147 is only one of the subunits of γ-secretase, other proteins are required for the formation of γ-secretase. Furthermore, to increase the extracellular Aβ level, they have to reduce CD147 level by at least 70%, indicating that a relatively low expression level of CD147 is sufficient for maintaining the function of γ-secretase [20]. In our study, hypoxia increased the expression of CD147. Since CD147 is a multi-functional protein, the augmented CD147 during hypoxia might exert other functions rather than assembling γ-secretase. Knockdown of excessive CD147 under hypoxia might also have no effect on the activity of γ-secretase. Further studies are required to reveal how excessive CD147 regulate Aβ expression under hypoxia.

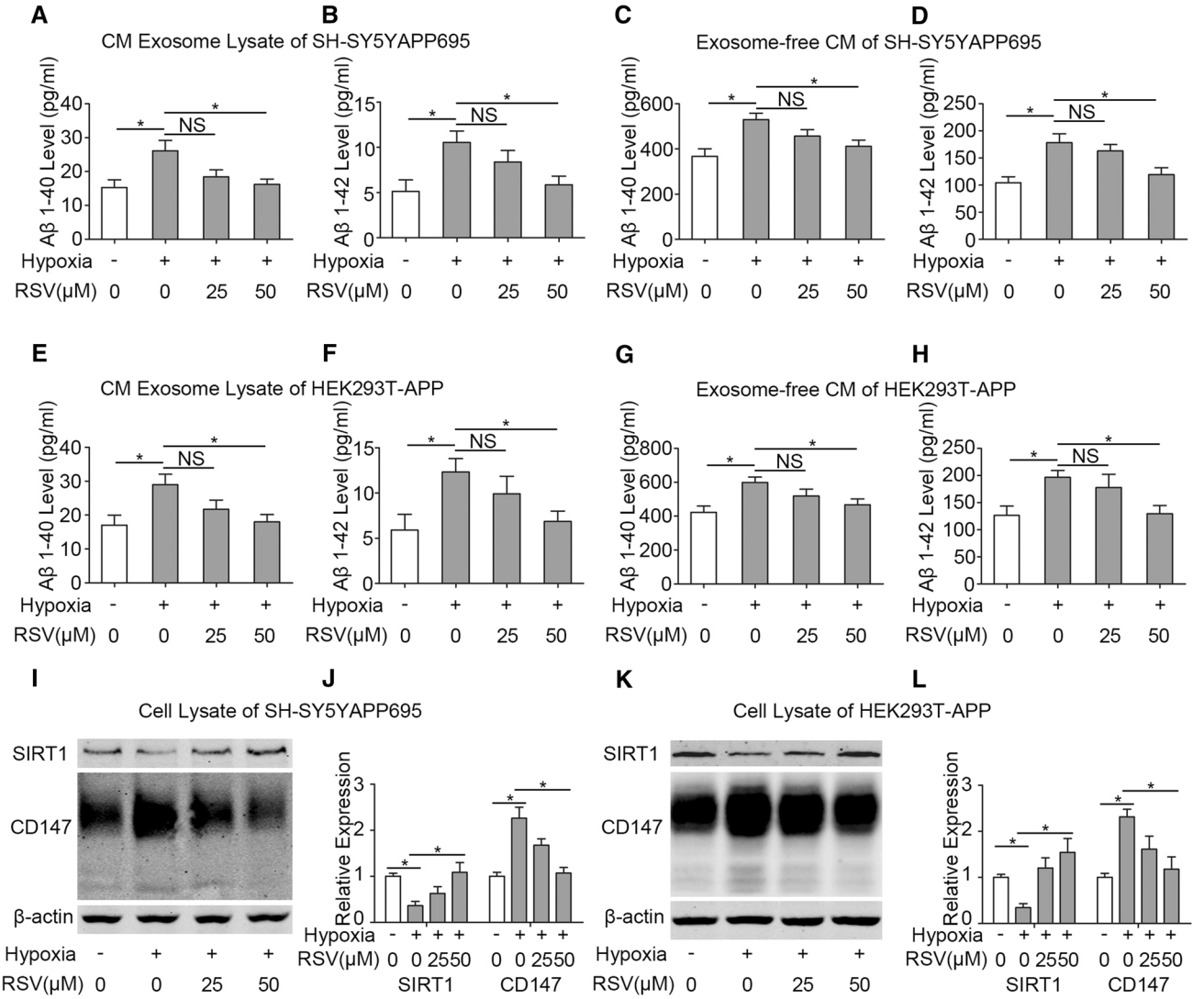


Fig. 3 RSV alleviated hypoxia-induced Aβ production and CD147 expression in vitro (n = 4). A-D,K-L: SH-SY5YAPP^{695} and HEK293T-APP cells were pretreated with 0, 25, and 50 μM RSV for 1 h and were then exposed to normoxia or hypoxia (2% O_2) for 24 h. The expression of Aβ1-40 and Aβ1-42 in cells-derived exosomal lysate (**a, b, e, f**) and exosome-free CM (**c, d, g, h**) was measured by ELISA. The expression of CD147 in cell lysate was detected by western blot (**i–l**). *represents $p < 0.05$

Previous studies showed that RSV could regulate CD147 expression through P38, ERK1/2 and PPARγ signaling pathways in monocytes, macrophages and vascular smooth muscle cells [22–24]. Moreover, another nature anti-oxidant polyphenol compound–green tea polyphenol epigallocatechin-3-gallate was shown to effectively inhibit CD147 expression through 67-kDa laminin receptor in PMA-induced macrophages [37]. In consistent with these studies, we found that RSV inhibited hypoxia induced CD147 expression both in vitro and in vivo. Inhibition of excessive CD147 further abrogated both total and exosomal Aβ production.

In conclusion, RSV abrogated hypoxia-induced up-regulation of total and exosomal Aβ partially by inhibiting CD147. Since RSV is an innoxious natural agent, the results would be of great value in the prevention and treatment of AD, especially under hypoxic conditions.

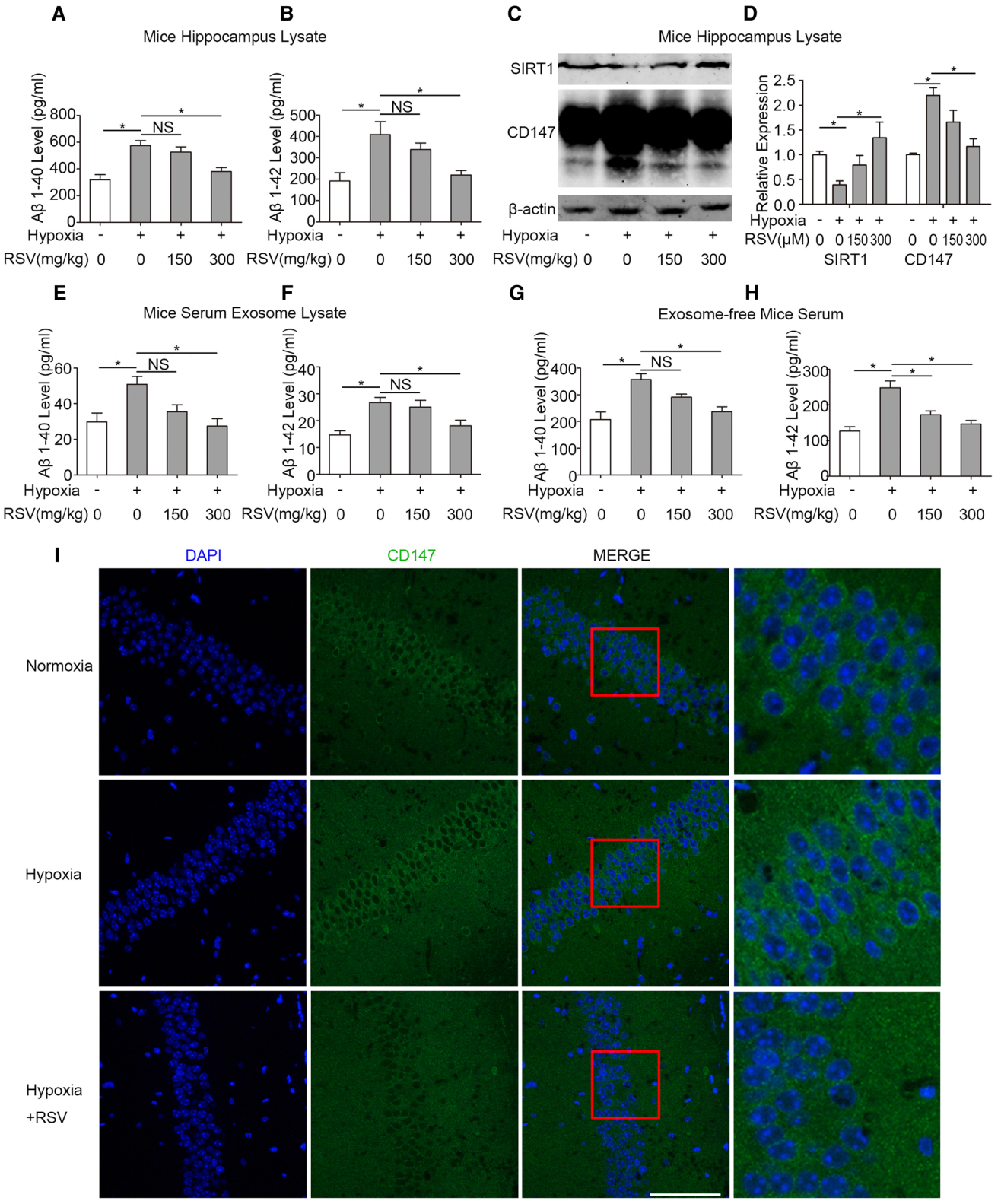


Fig. 4 RSV alleviated hypoxia-induced Aβ production and CD147 expression in vivo (n = 6). C57BL/6 mice were fed with normal diet or diets supplemented with 150 or 300 mg/kg RSV for 4 weeks and were then exposed to normoxia or hypoxia (8% O_2) for 5 h. The expression of Aβ1–40 (**a, e, g**) and Aβ1–42 (**b, f, h**) in hippocampus tissue lysate (**a, b**), mice serum-derived exosomal lysate (**e, f**), and exosome-free serum (**g, h**) were measured by ELISA. The expression of SIRT1 and CD147 in hippocampus tissue was detected by western blot (**c, d**). The expression of CD147 in hippocampus tissue of nomoxia, hypoxia and 300 mg/kg RSV fed mice were detected by immunofluorescence (**i**). *represents $p < 0.05$, Bar:100 μm

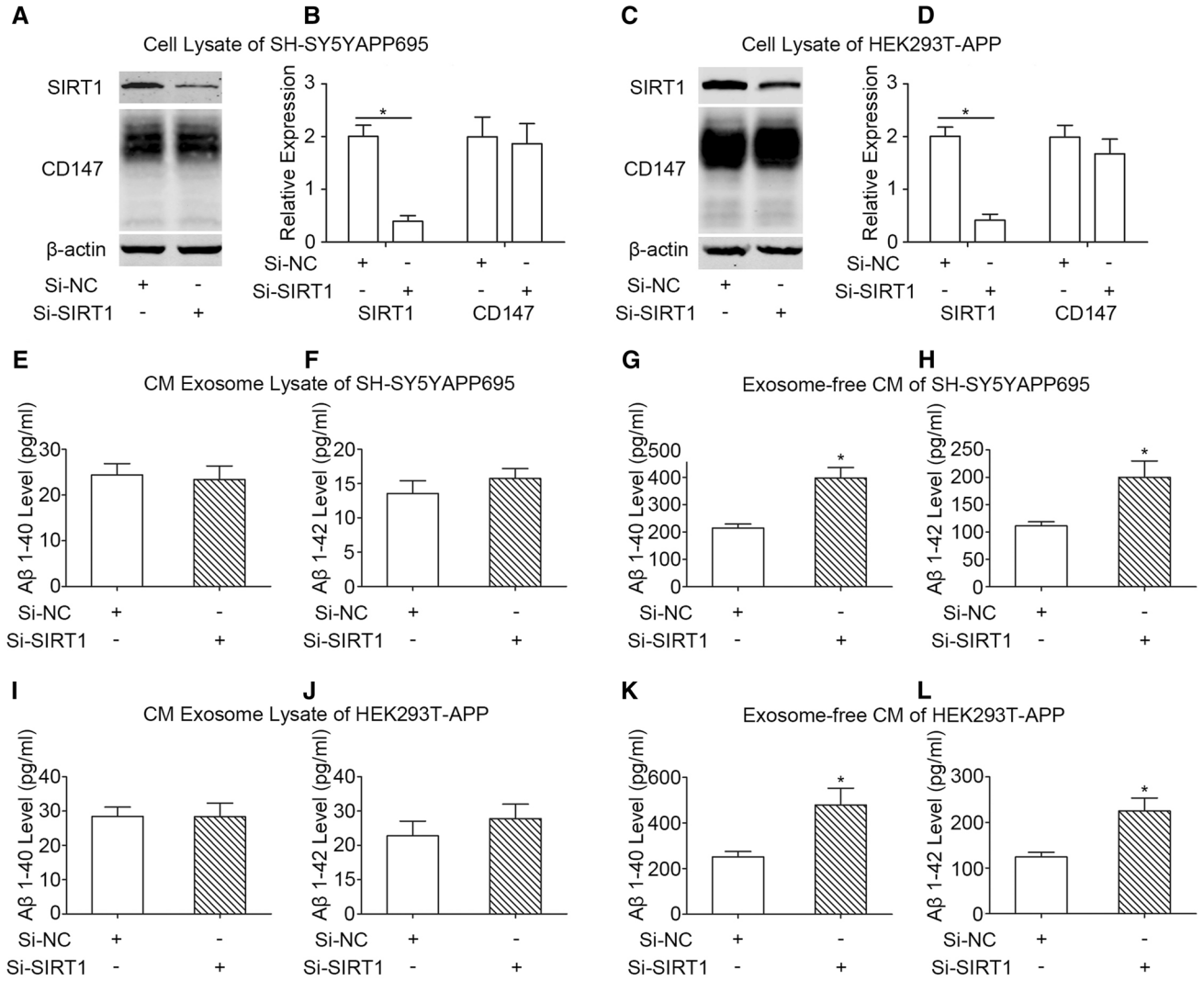


Fig. 5 Knocking down SIRT1 increased Aβ level in exosome-free CM but not in exosomal lysate (n = 4). SH-SY5YAPP^{695} and HEK293T-APP cells were transfected with negative control siRNA (si-NC) or SIRT1-targeting siRNA (si-SIRT1) for 24 h, and were then exposed to hypoxic condition (2% O_2) for 24 h. The expression of SIRT1 and CD147 was detected by western blot (**a**–**d**). Exosomes extracted from CM (**e, f, i, j**) were re-suspended in 100 ul diluent buffer and were sonicated. Exosome-free CM was collected during the exosome-extraction procedure (**g, h, k, l**). Aβ1-40 (**e, I, g, k**) and Aβ1-42 (**f, h, j ,l**) levels were measured by ELISA. *represents $p < 0.05$

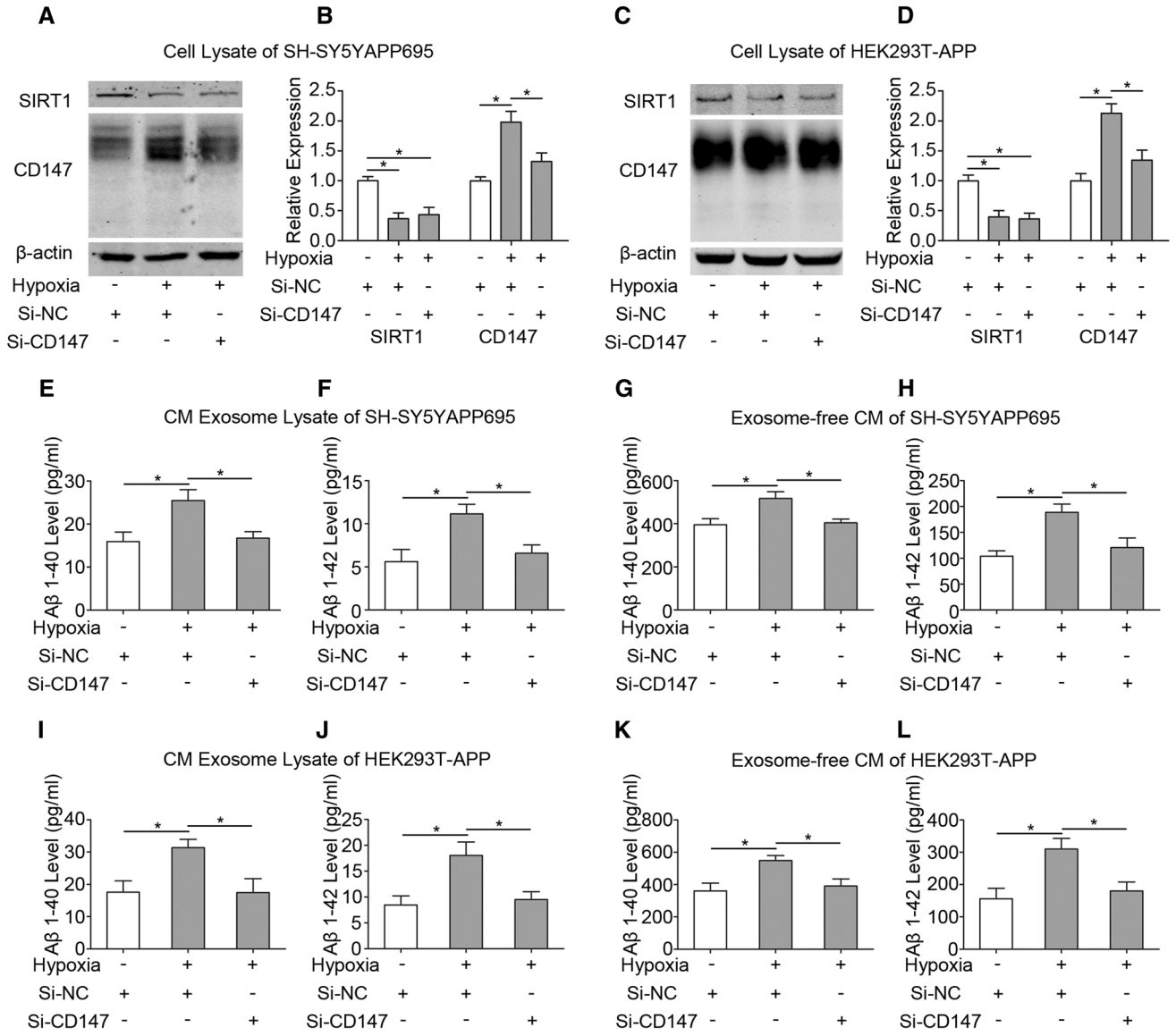


Fig. 6 Hypoxia induced Aβ augmentation in exosomes was mediated by increased expression of CD147 (n = 4). SH-SY5YAPP^{695} and HEK293T-APP cells were transfected with negative control siRNA (si-NC) or CD147-targeting siRNA (siCD147) for 24 h, and were then exposed to hypoxic condition (2% O_2) for 24 h. The expression of SIRT1 and CD147 in was detected by western blot (**a–d**). Exosomes extracted from CM (**e, f, i, j**) were re-suspended in 100 ul diluent buffer and were sonicated. Exosome-free CM was collected during the exosome-extraction procedure (**g, h, k, l**). Aβ1-40 (**e, g, i, k**) and Aβ1-42 (**f, h, j, l**) levels were measured by ELISA. *represents $p < 0.05$

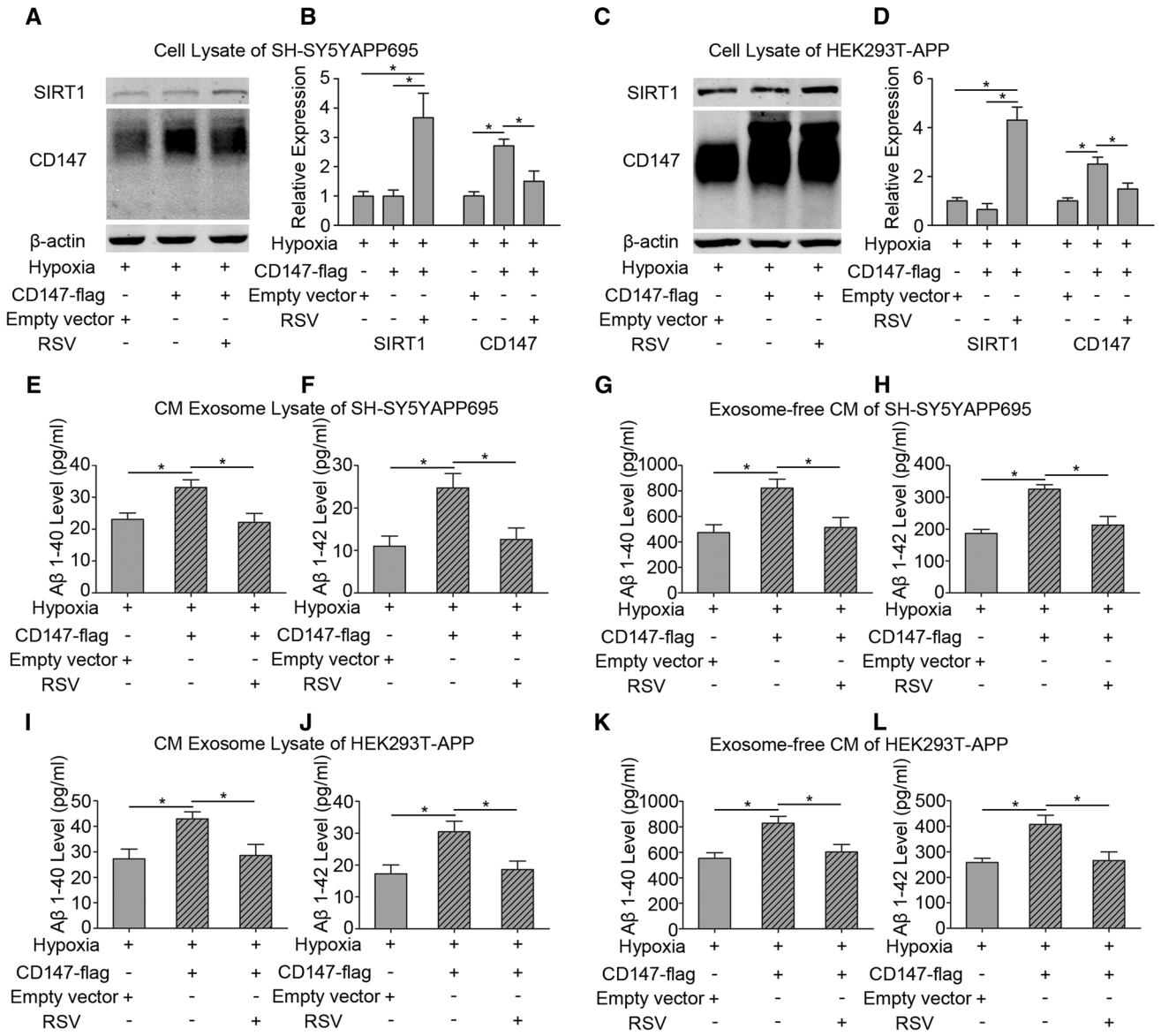


Fig. 7 Under hypoxic condition, RSV abrogated CD147-overexpression -induced augmentation of both total and exosomal Aβ production (n = 4). SH-SY5YAPP^{695} and HEK293T-APP cells were transfected with plasmid overexpressing CD147 (GV208-CD147-flag) or control plasmid empty vector (EV). After 24 h, cells were pretreated with 50 μM RSV or vehicle for 1 h and were then exposed to normoxia or hypoxia (2% O_2) for another 24 h. **a–d** The expression of SIRT1 and CD147 in cells was detected by western blot. **c–f** The expression of Aβ1–40 (**e, g, i, k**) and Aβ1–42 (**f, h, j, l**) in cells derived exosomal lysate (**e, f, i, j**) and exosome-free CM (**g, h, k, l**) was measured by ELISA. *represents $p < 0.05$

Acknowledgements This work was supported by the National Natural Science Foundation of China (grant numbers 81571033 and 81771131), Science and Technology Commission of Shanghai Municipality (No. 14DZ1930803, No. 18140901900 and No. 17411950101), and Climbing Talent Program of Shanghai Tenth People's Hospital (2018SYPDRC044).

Author Contributions JCX and XYL conducted most of the experiments and wrote the draft of the manuscript. Yu-Chen Zhou, XYM and Yi-Chen Zhao participate in the animal experiments. JYW and YT participate in data analysis and revised the manuscript. YXZ and XYL designed the experiments and supervised this project. All authors approved the final submitted version of the manuscript.

Compliance with Ethical Standards

Conflict of interest The authors declare no conflict of interest in this paper.

References

1. LaFerla FM, Green KN, Oddo S (2007) Intracellular amyloid-beta in alzheimer's disease. Nat Rev Neurosci 8:499–509
2. Haass C, Selkoe DJ (1993) Cellular processing of beta-amyloid precursor protein and the genesis of amyloid beta-peptide. Cell 75:1039–1042
3. Rajendran L, Honsho M, Zahn TR, Keller P, Geiger KD, Verkade P, Simons K (2006) Alzheimer's disease beta-amyloid peptides are released in association with exosomes. Proc Natl Acad Sci USA 103:11172–11177
4. Tkach M, Thery C (2016) Communication by extracellular vesicles: where we are and where we need to go. Cell 164:1226–1232
5. Kokubo H, Saido TC, Iwata N, Helms JB, Shinohara R, Yamaguchi H (2005) Part of membrane-bound Abeta exists in rafts within senile plaques in Tg2576 mouse brain. Neurobiol Aging 26:409–418
6. Vingtdeux V, Sergeant N, Buee L (2012) Potential contribution of exosomes to the prion-like propagation of lesions in alzheimer's disease. Front Physiol 3:229
7. Bellingham SA, Guo BB, Coleman BM, Hill AF (2012) Exosomes: vehicles for the transfer of toxic proteins associated with neurodegenerative diseases? Front Physiol 3:124
8. Farkas E, Luiten PG (2001) Cerebral microvascular pathology in aging and alzheimer's disease. Prog Neurobiol 64:575–611
9. Chui HC, Ramirez-Gomez L (2015) Clinical and imaging features of mixed alzheimer and vascular pathologies. Alzheimer's Res Ther 7:21
10. Li L, Zhang X, Yang D, Luo G, Chen S, Le W (2009) Hypoxia increases Abeta generation by altering beta- and gamma-cleavage of APP. Neurobiol Aging 30:1091–1098
11. Peers C, Dallas ML, Boycott HE, Scragg JL, Pearson HA, Boyle JP (2009) Hypoxia and neurodegeneration. Ann N Y Acad Sci 1177:169–177
12. Sun X, He G, Qing H, Zhou W, Dobie F, Cai F, Staufenbiel M, Huang LE, Song W (2006) Hypoxia facilitates Alzheimer's disease pathogenesis by up-regulating BACE1 gene expression. Proc Natl Acad Sci USA 103:18727–18732
13. Bu XL, Liu YH, Wang QH, Jiao SS, Zeng F, Yao XQ, Gao D, Chen JC, Wang YJ (2015) Serum amyloid-beta levels are increased in patients with obstructive sleep apnea syndrome. Sci Rep 5:13917
14. Xie JC, Ma XY, Liu XH, Yu J, Zhao YC, Tan Y, Liu XY, Zhao YX (2018) Hypoxia increases amyloid-beta level in exosomes by enhancing the interaction between CD147 and Hook1. Am J Transl Res 10:150–163
15. Chiang MC, Nicol CJ, Cheng YC (2018) Resveratrol activation of AMPK-dependent pathways is neuroprotective in human neural stem cells against amyloid-beta-induced inflammation and oxidative stress. Neurochem Int 115:1–10
16. Sadhukhan P, Saha S, Dutta S, Mahalanobish S, Sil PC (2018) Nutraceuticals: an emerging therapeutic approach against the pathogenesis of alzheimer's disease. Pharmacol Res 129:100–114
17. Wang R, Zhang Y, Li J, Zhang C (2017) Resveratrol ameliorates spatial learning memory impairment induced by Abeta1-42 in rats. Neuroscience 344:39–47
18. Jia Y, Wang N, Liu X (2017) Resveratrol and amyloid-beta: mechanistic insights. Nutrients 10:9
19. Hu J, Lin T, Gao Y, Xu J, Jiang C, Wang G, Bu G, Xu H, Chen H, Zhang YW (2015) The resveratrol trimer miyabenol C inhibits beta-secretase activity and beta-amyloid generation. PloS ONE 10:e0115973
20. Zhou S, Zhou H, Walian PJ, Jap BK (2005) CD147 is a regulatory subunit of the gamma-secretase complex in Alzheimer's disease amyloid beta-peptide production. Proc Natl Acad Sci USA 102:7499–7504
21. Godoy JA, Rios JA, Zolezzi JM, Braidy N, Inestrosa NC (2014) Signaling pathway cross talk in alzheimer's disease. Cell Commun Signal 12:23
22. Venkatesan B, Valente AJ, Reddy VS, Siwik DA, Chandrasekar B (2009) Resveratrol blocks interleukin-18-EMMPRIN cross-regulation and smooth muscle cell migration. Am J Physiol Heart Circ Physiol 297:H874–H886
23. Huang Z, Wang C, Wei L, Wang J, Fan Y, Wang L, Wang Y, Chen T (2008) Resveratrol inhibits EMMPRIN expression via P38 and ERK1/2 pathways in PMA-induced THP-1 cells. Biochem Biophys Res Commun 374:517–521
24. Ge H, Zhang JF, Guo BS, He Q, Wang BY, He B, Wang CQ (2007) Resveratrol inhibits macrophage expression of EMMPRIN by activating PPARgamma. Vasc Pharmacol 46:114–121
25. Cheang WS, Wong WT, Wang L, Cheng CK, Lau CW, Ma RCW, Xu A, Wang N, Huang Y, Tian XY (2018) Resveratrol ameliorates endothelial dysfunction in diabetic and obese mice through sirtuin 1 and peroxisome proliferator-activated receptor delta. Pharmacol Res 139:384–394
26. Corpas R, Grinan-Ferre C, Rodriguez-Farre E, Pallas M, Sanfeliu C (2018) Resveratrol induces brain resilience against alzheimer neurodegeneration through proteostasis enhancement. Mol Neurobiol. https://doi.org/10.1007/s12035-018-1157-y
27. Karuppagounder SS, Pinto JT, Xu H, Chen HL, Beal MF, Gibson GE (2009) Dietary supplementation with resveratrol reduces plaque pathology in a transgenic model of alzheimer's disease. Neurochem Int 54:111–118
28. Ding S, Jiang J, Wang Z, Zhang G, Yin J, Wang X, Wang S, Yu Z (2018) Resveratrol reduces the inflammatory response in adipose tissue and improves adipose insulin signaling in high-fat diet-fed mice. PeerJ 6:e5173
29. Alzheimer's Association (2016) 2016 Alzheimer's disease facts and figures. Alzheimer's Dement 12:459–509
30. Bloom GS (2014) Amyloid-beta and tau: the trigger and bullet in alzheimer disease pathogenesis. JAMA Neurol 71:505–508
31. Ma L, Fu R, Duan Z, Lu J, Gao J, Tian L, Lv Z, Chen Z, Han J, Jia L, Wang L (2016) Sirt1 is essential for resveratrol enhancement of hypoxia-induced autophagy in the type 2 diabetic nephropathy rat. Pathol Res Pract 212:310–318
32. Nahalkova J, Volkmann I, Aoki M, Winblad B, Bogdanovic N, Tjernberg LO, Behbahani H (2010) CD147, a gamma-secretase associated protein is upregulated in Alzheimer's disease brain and its cellular trafficking is affected by presenilin-2. Neurochem Int 56:67–76
33. Marimpietri D, Petretto A, Raffaghello L, Pezzolo A, Gagliani C, Tacchetti C, Mauri P, Melioli G, Pistoia V (2013) Proteome profiling of neuroblastoma-derived exosomes reveal the expression of proteins potentially involved in tumor progression. PloS ONE 8:e75054
34. Vetrivel KS, Zhang X, Meckler X, Cheng H, Lee S, Gong P, Lopes KO, Chen Y, Iwata N, Yin KJ, Lee JM, Parent AT, Saido TC, Li YM, Sisodia SS, Thinakaran G (2008) Evidence that CD147 modulation of beta-amyloid (Abeta) levels is mediated by extracellular degradation of secreted Abeta. J Biol Chem 283:19489–19498
35. Kaur B, Khwaja FW, Severson EA, Matheny SL, Brat DJ, Van Meir EG (2005) Hypoxia and the hypoxia-inducible-factor pathway in glioma growth and angiogenesis. Neuro-Oncology 7:134–153
36. Ashok A, Rai NK, Raza W, Pandey R, Bandyopadhyay S (2016) Chronic cerebral hypoperfusion-induced impairment of Abeta clearance requires HB-EGF-dependent sequential activation of HIF1alpha and MMP9. Neurobiol Dis 95:179–193

37. Wang QM, Wang H, Li YF, Xie ZY, Ma Y, Yan JJ, Gao YF, Wang ZM, Wang LS (2016) Inhibition of EMMPRIN and MMP-9 Expression by Epigallocatechin-3-Gallate through 67-kDa Laminin Receptor in PMA-Induced Macrophages. Cell Physiol Biochem 39:2308–2319