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Original Article

Hypoxia increases amyloid-β level in exosomes by enhancing the interaction between CD147 and Hook1

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Abstract: Hypoxia promotes the accumulation of amyloid-β (Aβ), which is related to the pathogenesis of Alzheimer's disease (AD). CD147 is considered as an additional subunit of γ-secretase regulated by hypoxia, and has been identified in exosomes. Aβ is also found in exosomes that participate in the intercellular communication and amyloids propagation. This study was to investigate the role of CD147 in hypoxia-induced accumulation of Aβ in exosomes. Our results showed that hypoxia increased the levels of Aβ40 and Aβ42 in exosomes and enhanced the interaction between CD147 and Hook1 in SH-SY5YAPP^{695} cells. Moreover, hypoxia increased the interaction between amyloid precursor protein (APP) and CD147 as well as the expression of CD147 in isolated membrane. After we interfered the interaction between CD147 and Hook1 by decreasing Rab22a expression, the hypoxia induced Aβ accumulation in exosomes was significantly suppressed. In addition, the increased interaction between CD147 and Hook1 was further confirmed in hypoxia exposed C57BL/6 mice. Our findings reveal that hypoxia may increase exosome Aβ level by enhancing the interaction between CD147 and Hook1.

Keywords: Amyloid-β, exosome, hypoxia, CD147, Hook1

Introduction

The amyloid plaques and neurofibrillary tangles have been known as the histopathological signatures of Alzheimer disease (AD) since the early 20th century [1]. Extracellular amyloid plaques and intraneuronal neurofibrillary tangles have highly insoluble, densely packed filaments, which are formed by amyloid-β (Aβ) peptides for plaques and tau for tangles, and their accumulation directly leads to the impairment and destruction of synapses related to memory and cognition [1]. The hydrophobic 39-43 residue Aβ is proteolytically derived from the transmembrane amyloid precursor protein (APP) [2] after sequential cleavage by β-secretase and γ-secretase [3].

A variety of studies have revealed that hypoxia is able to augment the amyloidogenic processing of APP, resulting in the Aβ accumulation [4, 5]. It has been reported that hypoxia can modify APP processing and enhance the activity of β-site APP-cleaving enzyme 1 (BACE1) and PS1/γ-secretase complex, which accelerates Aβ production and plaques formation *in vivo* and *in vitro* [6].

Aβ peptides have been found in multivesicular bodies (MVBs) and can be released from exosomes, which are a type of microvesicles with the diameters of 40-100 nm and can be secreted after MVBs fusion with the cell surface [7]. In addition, APP, APP-CTFs and AICD can also be integrated and secreted via exosomes in neuronal cells [8, 9]. Exosomes containing amyloidogenic proteins and peptides as intercellular shuttles may act as mediators of amyloids propagation [10, 11], and the presence of exosome markers in amyloid plagues of Tg2576 mice brains and post-mortem human AD patients indicates that exosomes may affect the trafficking of Aβ and their aggregation to plaques [12, 13], which may be partially related to the progression of AD.

Nevertheless, the effects of hypoxia on the Aβ accumulation in exosomes and the specific mechanisms are still unclear. CD147, a transmembrane glycoprotein, has been regarded as

an additional subunit of the γ-secretase complex and may influence the Aβ accumulation [14]. CD147 was also identified in neuroblastoma cell-derived exosome [15]. Our results showed that hypoxia could up-regulate the CD147 expression in SH-SY5YAPP^{695} cells. Furthermore, the microtubule- and cargo-tethering protein, Hook1, has been found to recognize the cytoplasmic tail of CD147 and help sort it into Rab22a-dependent tubules associated with recycling [16]. This means that Hook1 functions as an adaptor that selectively sorts CD147 on endosomes to prevent its trafficking from the degradation route. However, the roles of CD147 and Hook1 in the regulation of Aβ production in exosomes and the changes in CD147 and Hook1 under hypoxic conditions remain inconclusive. This study aimed to investigate the effects of hypoxia on the Aβ accumulation in exosomes and the roles of CD147 and Hook1 in these effects.

Material and methods

Cell lines and hypoxia exposure

Human neuroblastoma SH-SY5Y cells with stable expression of human wild-type APP^{695} cDNA (SH-SY5YAPP^{695}) were kindly provided by Professor Sheng-Di Chen in the Department of Neurology and Institute of Neurology, Ruijin Hospital, Shanghai JiaoTong University School of Medicine, Shanghai. For hypoxia exposure, SH-SY5YAPP^{695} cells were exposed to 2% O_2 for 24 h, and the plates were placed in the chamber (Thermo Scientific™ Forma™ Steri-Cycle™ i60 CO_2), meanwhile the control plates were placed in the other incubator with a mixed gas of 21% O_2, 5% CO_2, and balance N_2.

Reagents, siRNA, plasmids and transfection

The following primary antibodies were used: anti-Hook1 antibody (rabbit, 1:2000 for WB and 1:50 for co-immunoprecipitation, Sigma-Aldrich, HPA018537), anti-CD147 antibody (mouse, 1:1000 for WB and 1:50 for co-immunoprecipitation, SantaCruz Biotechnology, sc-25-273), anti-CD147 antibody (rabbit, 1:1000 for WB, Abcam, ab108317), anti-APP antibody (rabbit, 1:1000 for WB and 1:50 for co-immunoprecipitation, Abcam, ab32136), anti-Rab22a antibody (rabbit, 1:500 for WB, Abcam, ab-137093), anti-ALIX antibody (rabbit, 1:1000 for WB, Sigma-Aldrich, SAB4200477), anti-Flotillin 1 antibody (rabbit, 1:1000 for WB, Abcam, ab41927), Anti-alpha 1 Sodium Potassium ATPase (mouse, 1:1000 for WB, Abcam, USA ab7671). Membrane and Cytosol Protein Extraction Kit (Beyotime® Biotechnology, China #P0033) was used for membrane protein extraction.

In some experiments, siRNA or plasmids transfection of SH-SY5YAPP^{695} cells was performed using the Lipofectamine® 3000 kit (Invitrogen, Life Technologies) according to the manufacturer's instructions. The Rab22a siRNA were synthesized in Genepharma (Shanghai, China). Plasmid over-expressing Hook1 (HA-Hook1) was constructed in GeneChem Corporation (Shanghai, China).

Real-time PCR

Total RNAs were extracted with a TRIzol reagent kit, and reverse transcription of RNA into cDNA was done using the PrimeScript™ RT reagent Kit (Takara Clontech, Kyoto, Japan). Real-time PCR was performed using the SuperReal PreMix Plus (SYBR Green) kit (Takara Clontech, Kyoto, Japan) on Roche LightCycler480 System. The reaction mixture (20 μl) contained 10 μl of 2 × SYBR® Premix Ex Taq™, 500 nM oligonucleotide primers (Shanghai Generay Biotech Co., Ltd China), and 0.5 μl of cDNA. The thermal cycling conditions included pre-denaturation at 95°C for 30 s and 40 cycles of 95°C for 5 s and 60°C for 30 s. The mRNA expression of target genes was normalized to that of β-actin.

Western blotting

After treatment, SH-SY5YAPP^{695} cells were lysed in RIPA buffer (Beyotime, Shanghai, China) supplemented with Halt Protease Inhibitor Cocktail (Thermo Scientific, Rockford, USA) on ice for 30 min. The lysate was centrifuged at 12,000 rpm for 5 min, and total protein concentration was quantified by bicinchoninic acid assay (BCA) (Pierce, Rockford, IL, USA). Then, 60 μg of proteins was loaded and separated by 10% or 15% SDS-PAGE and then transferred onto nitrocellulose membrane (Bio-rad, CA, USA). The membranes were blocked in TBS containing 5% bovine serum albumin (BSA, Sigma Aldrich, MO, USA) for 1 h at room temperature. The membranes were then incubated with primary antibodies at 4°C over-

night. After rinsing thrice, the membranes were incubated with Mouse IgG (H&L) Antibody IRDye800® Conjugated and Rabbit IgG (H&L) Antibody IRDye700® Conjugated (Li Cor Biosciences, NE, USA) at room temperature for 1 h. After washing thrice, the protein bands were detected with the Odyssey infrared imaging system (Li Cor Biosciences, NE, USA).

Immunofluorescent staining

SH-SY5YAPP^{695} cells were seeded and cultured on coverslips in 24-well plates. After hypoxic exposure for 24 h, cells were washed and fixed in cold acetone for 5 min. After washing with PBS thrice, cells were blocked with 2% BSA for 1 h at room temperature and incubated with primary antibodies overnight at 4°C. Primary antibodies were anti-HOOK1 antibody (rabbit, 1:50, Sigma-Aldrich, St. Louis, MO, USA HPA018537) and anti-CD147 antibody (mouse, 1:50, SantaCruz Biotechnology, CA, USA sc-25273). After rinsing with PBS thirce, cells on the coverslips were further incubated with FITC-conjugated secondary antibodies for 1 h at room temperature. Secondary antibodies were Dylight 488 AffiniPure goat anti-mouse IgG and CY3 AffiniPure goat anti-rabbit IgG (all 1:200, Jackson Immunoresearch Laboratories, Inc). After counterstaining with 4,6-diamidino-2-phenylindole (DAPI), mounting was performed, and cells were visualized under a confocal microscope (Carl Zeiss, LSM710, Jena, Germany).

Co-immunoprecipitation assay

SH-SY5YAPP^{695} cells were harvested from 10-cm dishes, sonicated in the lysis/wash buffer and incubated with 10 μl of anti-Hook1 antibody, anti-CD147 antibody or anti-APP antibody overnight at 4°C. Co-immunoprecipitation was performed with Pierce Classic Magnetic IP/Co-IP kit according to the manufacturer's instructions. Cell lysate and immunoprecipitate were analyzed by Western blotting.

To detect the interaction between CD147 and Hook1 in C57BL/6 mice, the hippocampus was collected, weighed, homogenized in 100 μl of ice-cold lysis/wash buffer containing protease inhibitors with Dounce Homogenizers and then sonicated. After centrifugation at 4°C, the resultant supernatant was collected and quantified with a BCA kit (Pierce, Rockford, IL, USA). Thereafter, co-immunoprecipitation was performed as described above.

Isolation of exosomes

Cells were incubated in culture medium with exosome-depleted FBS (Gibco, Thermo Fisher Scientific) for 48 h, and then exosomes were isolated from the medium. Conditioned medium was collected, and then exosomes were precipitated with ExoQuick exosome precipitation solution (EXOQ-TC; System Biosciences, Inc., Mountain View, CA, USA). Briefly, 4 ml of ExoQuick reagent was added into 20 ml of conditioned medium with inhibitor cocktails, followed by refrigeration overnight. On the secondary day, the ExoQuick-TC/biofluid mixture was centrifuged at 1,500 × g for 30 min and the supernatant was aspirated. Then residual ExoQuick solution was centrifuged at 1,500 × g for 5 min and all traces of fluid were removed by aspiration carefully to precipitate exosomes according to the manufacturer's instructions.

ELISA of Aβ

Conditioned medium was collected from SH-SY5YAPP^{695} cells. Halt Protease Inhibitor Cocktail (Thermo Scientific, Rockford, USA) was added to the medium to prevent against Aβ degradation. The concentrations of Aβ40 and Aβ42 were determined with human Aβ40 and Aβ42 ELISA kits (Elabscience Biotechnology, Wuhan, China), respectively according to the manufacturer's instructions. Amyloid beta 42 Human ELISA Kit Ultrasensitive (Thermo Fisher Scientific) was used to detect exosomal Aβ42. Briefly, exosomes pellets were re-suspended in diluent buffer of ELISA kits, and sonicated, followed by quantification. The resultant solution was then added to the plate and ELISA was performed according to the manufacturer's instructions.

Animals and treatments

Male C57BL/6 mice aged 8-10 weeks (specific pathogen-free, SPF) were purchased from Shanghai SLAC Laboratory Animal Co. Ltd (Shanghai, China), housed in SPF environment and give *ad libitum* access to food and water. 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.

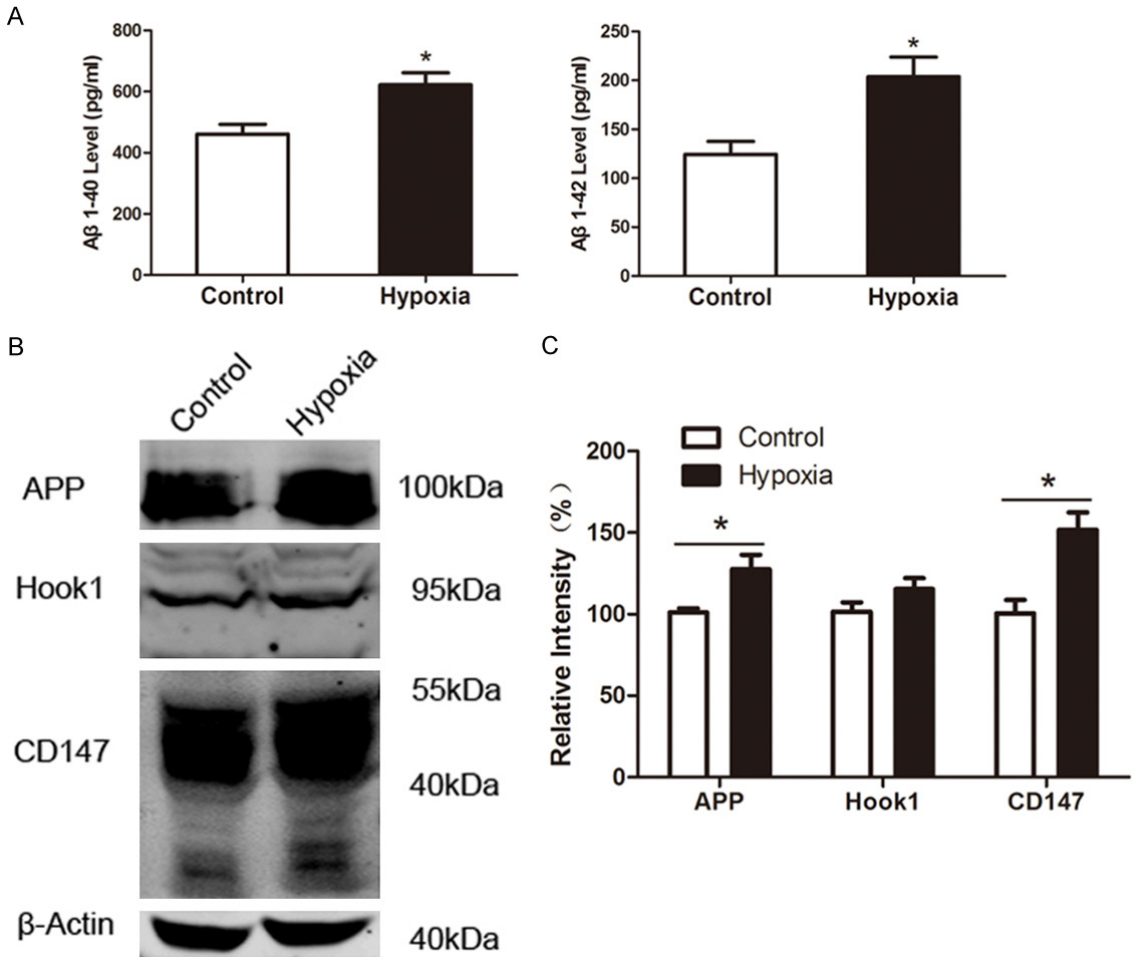

Figure 1. Hypoxia increases the expression of CD147 and APP and the level of Aβ in SH-SY5YAPP^{695} cells. A. SH-SY5YAPP^{695} cells were exposed to 2% O_2 for 24 h. The medium was collected and sonicated, and then Aβ40 and Aβ42 were measured by ELISA kit. *P<0.05. B, C. Hypoxia increased the expression of CD147 and APP, while Hook1 expression remained unchanged. SH-SY5YAPP^{695} cells were exposed to 2% O_2 for 24 h and then cell lysates were processed for Western blotting. β-actin served as an internal control. C. Densitometric analysis of APP, CD147 and Hook1 expression. Data are expressed as mean ± SEM (n = 3) *P<0.05.

Mice were exposed to 8% O_2 for 5 h in a specially designed hermetic chamber for animal experiments. In control group, mice were exposed to the air in the same chamber (21% O_2). Mice were sacrificed by decapitation after hypoxia exposure and the brains were promptly collected. Two hemispheres were obtained for Western blotting and co-immunoprecipitation assay, respectively. Hippocampus was quickly separated, weighed and homogenized in 5 volumes of ice-cold lysis buffer, or stored at -80°C before further processing. The homogenates were kept on ice for 30 min and then centrifuged at 12,000 rpm for 10 min at 4°C. Total protein concentration was quantified using a BCA kit (Pierce, Rockford, IL, USA).

Statistical analysis

Experiments were done at least three times. Data are expressed as means ± standard error (SEM). Comparisons were done with one-way analysis of variance (ANOVA) or two tailed Student's t test. A value of $P<0.05$ was considered statistically significant.

Results

Hypoxia increased Aβ level and up-regulated expression of CD147 and APP in SH-SY5Y-APP^{695} cells

The contents of Aβ40 and Aβ42 were detected in the culture medium from SH-SY5YAPP^{695}

cells. Results showed hypoxia increased the Aβ40 and Aβ42 contents from 461.3±31.98 pg/ml and 124.4±13.30 pg/ml to 622.4± 39.31 pg/ml and 203.9±19.95 pg/ml, respectively ($P<0.05$) (**Figure 1A**). Then, the expression of APP, CD147, and Hook1 in human SH-SY5YAPP^{695} cells was detected by Western blotting. As shown in **Figure 1B** and **1C**, hypoxia increased the expression of APP and CD-147 to 126.4% ($P = 0.0433$) and 150.6% ($P = 0.0192$), respectively, although significant change was not observed in Hook1 expression (113.3%; $P = 0.1925$). Our findings indicate that hypoxia increases Aβ production, which might be partially attributed to the increased expression of APP and CD147.

Hypoxia increased Aβ from exosomes

The extracted exosomes from SH-SY5YAPP^{695} cells were re-suspended in PBS and identified by electron microscope. Under an electron microscope, the exosomes had typical lipid bilayer structure with the diameter of 40-100 nm (**Figure 2A**). After hypoxia exposure, cells were lysed and the expression of well-characterized exosomal protein markers ALIX and Flotillin 1 was detected by Western blotting. The expression of Flotillin 1 and ALIX increased after hypoxia exposure (**Figure 2B**), which means hypoxia exposure increases the release of exosomes from cells.

We further investigated the influence of hypoxia exposure on the level of Aβ in exosomes. Cell medium was divided into two parts with equal volume and exosomes were extracted. One was used for protein quantitative analysis with BCA kit and the other was used for detecting Aβ concentration by ELISA. Next, to investigate the Aβ level of exosomes from the two groups, the samples were first normalized by the results of quantitative analysis and verified by western blot (**Figure 2C**). The mean levels of Aβ40 and Aβ42 in the hypoxia group (Aβ40: 20.57±2.281 pg/ml and Aβ42: 11.34±2.005 pg/ml) were significantly higher than in the control group (Aβ40: 13.82±0.804 pg/ml and Aβ42: 4.438±0.929 pg/ml) by ELISA (**Figure 2D**). Meanwhile, the Aβ level was measured in exosome-depleted conditioned medium. After isolation of exosomes, the conditioned medium was processed for the detection of Aβ40 and Aβ42 contents. Results showed the levels of Aβ40 and Aβ42 increased after hypoxia exposure (**Figure 2E**). These indicate that exosome-generated Aβ increases in SH-SY5-YAPP^{695} cells under hypoxic condition, although they are only a small part of the total extracellular Aβ.

Hypoxia increased the level of CD147 in the membrane of SH-SY5YAPP^{695} cells

APP and CD147 are transmembrane proteins and their expression increased after hypoxia exposure. To investigate the expression of CD147 in the membrane under hypoxic condition, the membrane was isolated from SH-SY5YAPP^{695} cells and the level of CD147 was detected by Western blotting. Results showed that CD147 expression significantly increased under hypoxic condition (**Figure 3A**, **3B**).

APP directly interacted with CD147 in SH-SY5YAPP^{695} cells

We speculated that there was potential interaction between APP and CD147. Thus, co-immunoprecipitation assay was conducted to further investigate the interaction between APP and CD147. First, APP was immunoprecipitated from cell lysate using the Lysis buffer from the co-IP kit. Results (**Figure 3C**, **3D**) showed that the combination of APP and CD-147 was clearly enhanced when cells were exposed to hypoxia. Then, CD147 was also immunoprecipitated, which confirmed the co-IP results that hypoxia increased the interaction between CD147 and APP. These results suggest the existence of a physiological complex between APP and CD147.

Hypoxia augmented the interaction between CD147 and Hook1

To investigate the interaction between CD147 and Hook1 under hypoxic condition, co-immunoprecipitation assay was performed. SH-SY5-YAPP^{695} cells were transfected with HA-Hook1, and then exposed to hypoxia for 24 h. The overexpression of full-length Hook1 (HA-Hook1) was confirmed by Western blotting and real-time PCR. Transfection with HA-Hook1 plasmid showed significant increase in Hook1 expression (**Figure 4A**). After hypoxia exposure, cells were lysed and prepared for co-immunoprecipitation assay. Results showed that the overexpressed full-length Hook1 and endogenous CD147 could be co-immunoprecipitated, which

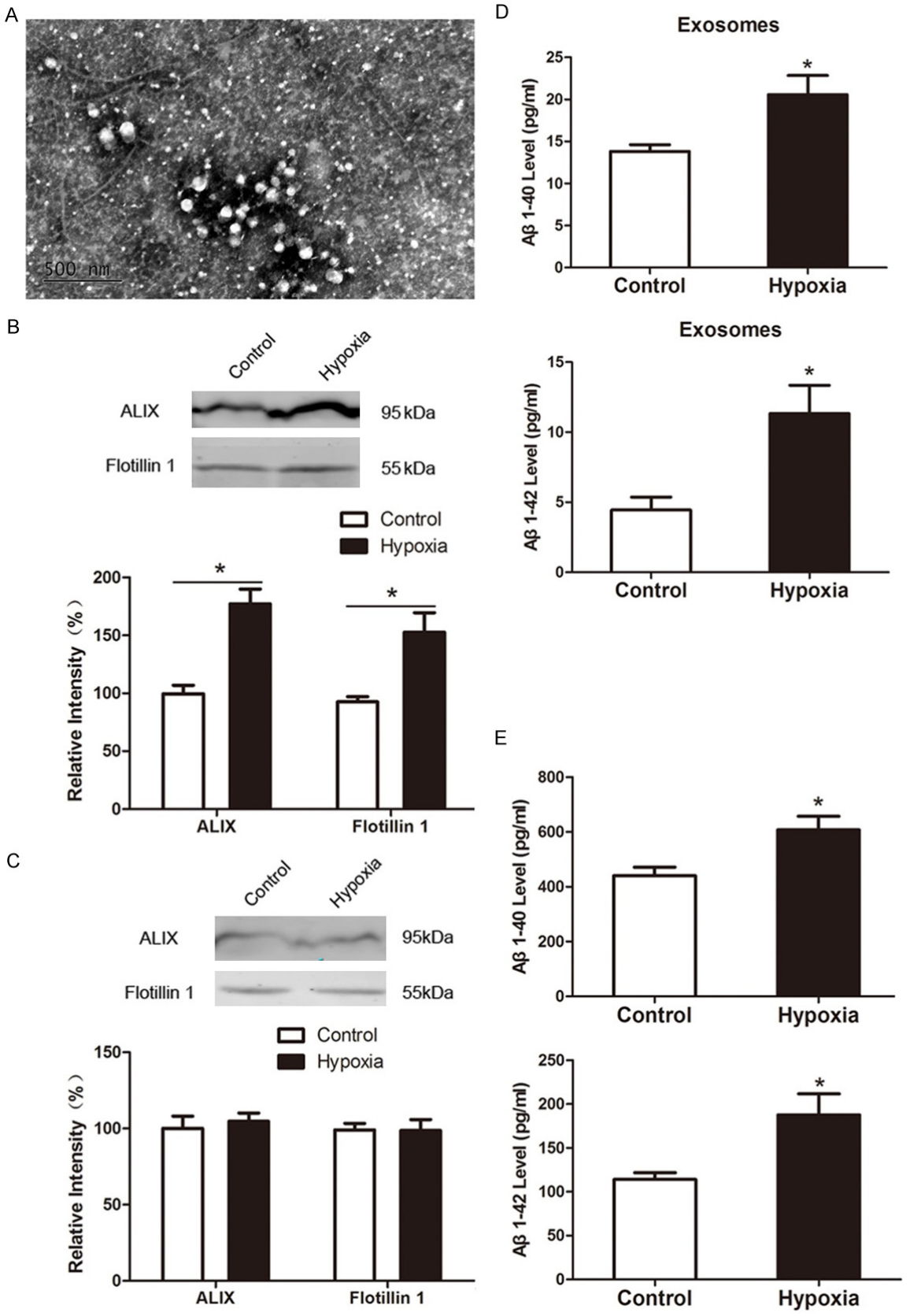

Figure 2. Hypoxia increases the level of Aβ in exosomes. A. Electron microscopic images of SH-SY5YAPP^{695} cells -derived exosomes. Exosomes were spherical vesicles surrounded by a bilayer lipid membrane and had the diameter

of between 40 and 100 nm. B. Hypoxia enhanced the release of exosomes in SH-SY5YAPP^{695} cells. Exosomes purified from conditioned medium were quantified by Western blotting using antigens ALIX and Flotillin 1. The protein expression of ALIX and Flotillin 1 was measured by densitometric analysis. C. Normalized exosomes from different groups. After quantitative analyses with BCA kit, exosomes from each group were adjusted to the same concentration and then Western blotting was performed. D. Hypoxia increased Aβ accumulation in exosomes. Aβ40 and Aβ42 were measured in exosomes at equal concentration by ELISA. E. Eliminating exosomes did not significantly influence the level of Aβ in conditioned medium. After isolation of exosomes, the remaining conditioned medium was also processed for the measurement of Aβ40 and Aβ42 by ELISA. Data are expressed as mean ± SEM (n = 3) *P<0.05.

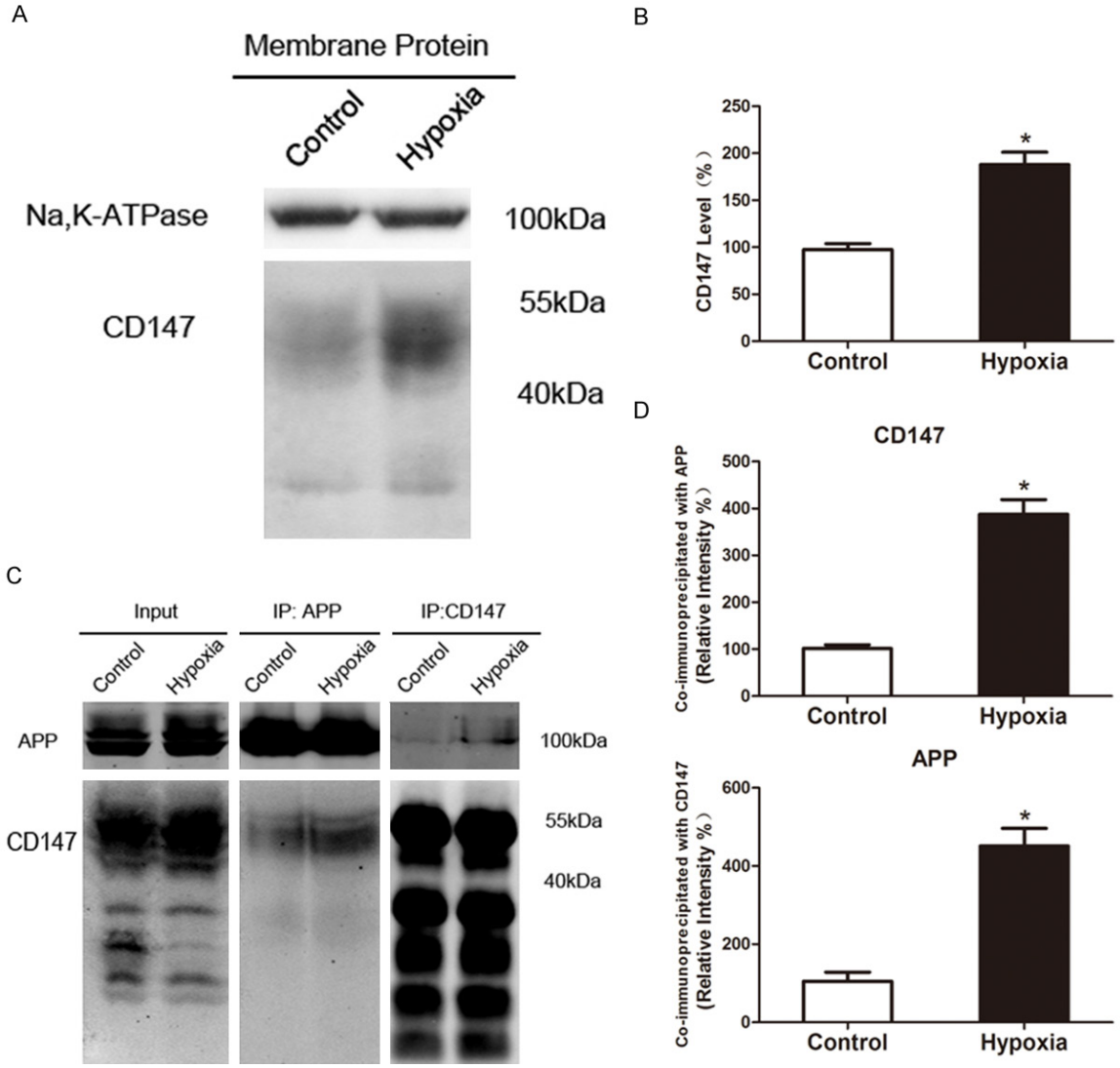

Figure 3. Hypoxia increases the interaction between APP and CD147 as well as the expression of CD147 in isolated membrane. A. CD147 expression in the membrane isolated from SH-SY5YAPP^{695} cells increases under hypoxic condition. The cell lysate was subjected to membrane isolation. Equal amounts of membranes lysates were immunoblotted with CD147 and Na^+/K^+-ATPase α1 antibodies. Na^+/K^+ ATPase was used as an internal control. B. Densitometric analysis of CD147 expression in the membrane. C. Direct interaction between CD147 and APP. SH-SY5YAPP^{695} cells were exposed to hypoxia for 24 h and then cell lysate was immunoprecipitated (IP) with indicated antibodies. Precipitated immunocomplexes were analyzed by Western blotting with anti-APP or anti-CD147 antibodies. Results showed the interaction between APP and CD147 was enhanced in hypoxia. D. Quantitative densitometric analysis was performed to assess co-IP between CD147 and APP following hypoxia exposure. Data are expressed as mean ± SEM (n = 3) *P<0.05.

was enhanced under hypoxic condition (**Figure 4B**, **4C**).

Besides, confocal microscopy supported the colocalization of CD147 and Hook1 in SH-SY5-

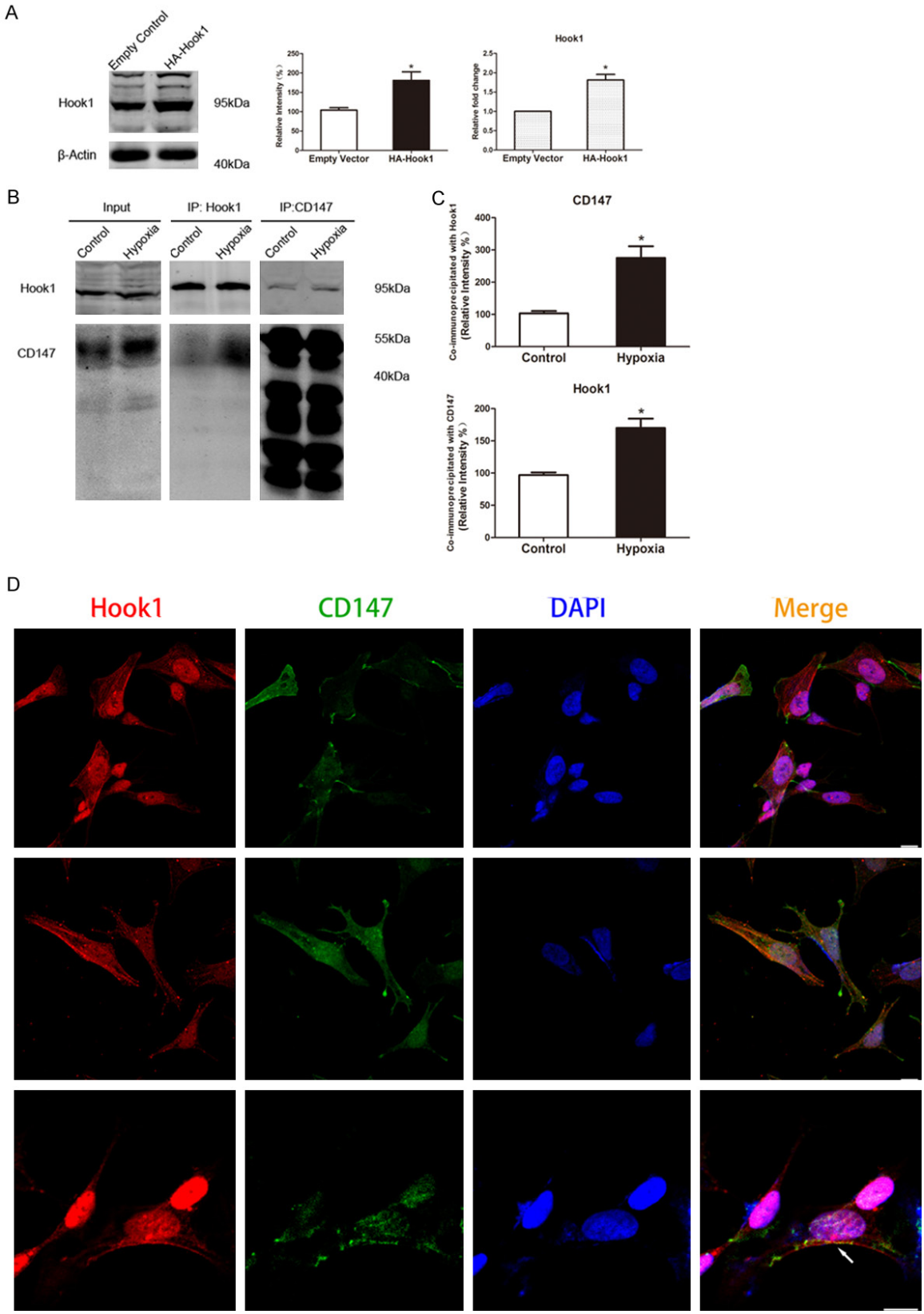

Figure 4. Hypoxia promotes the interaction between CD147 and Hook1. A. SH-SY5YAPP^{695} cells were transfected with HA-tagged full-length Hook1 (HA-Hook1) or empty vector. Western blotting and real-time PCR for Hook1 showed the expression of Hook1 was enhanced in cells transfected with HA-Hook1. B. Interaction between Hook1 and

CD147 (Co-IP). SH-SY5YAPP^{695} cells transfected with HA-Hook1 were exposed to hypoxia for 24 h and then cell lysate was subjected to Co-IP. Precipitated immunocomplexes were analyzed by Western blotting with anti-Hook1 or anti-CD147 antibodies. IP: Hook1 denotes Hook1-IP from cell lysates; IP: CD147 denotes CD147-IP from cell lysates. C. Quantitative densitometric analysis was performed to assess co-IP between CD147 and Hook1 following hypoxia exposure. Data are expressed as mean ± SEM (n = 3) *P<0.05. D. Co-localization of CD147 and Hook1 in SH-SY5YAPP^{695} cells exposed to hypoxia. Cells were fixed and subjected to double immunofluorescence staining with anti-Hook1 (red) and anti-CD147 (green) antibodies. Cells were counterstained with DAPI (blue). Upper row: SH-SY5YAPP^{695} cells in normoxia; middle and lower rows: cells in hypoxia. Arrow: CD147 and Hook1 colocalization. Bars: 10 μm.

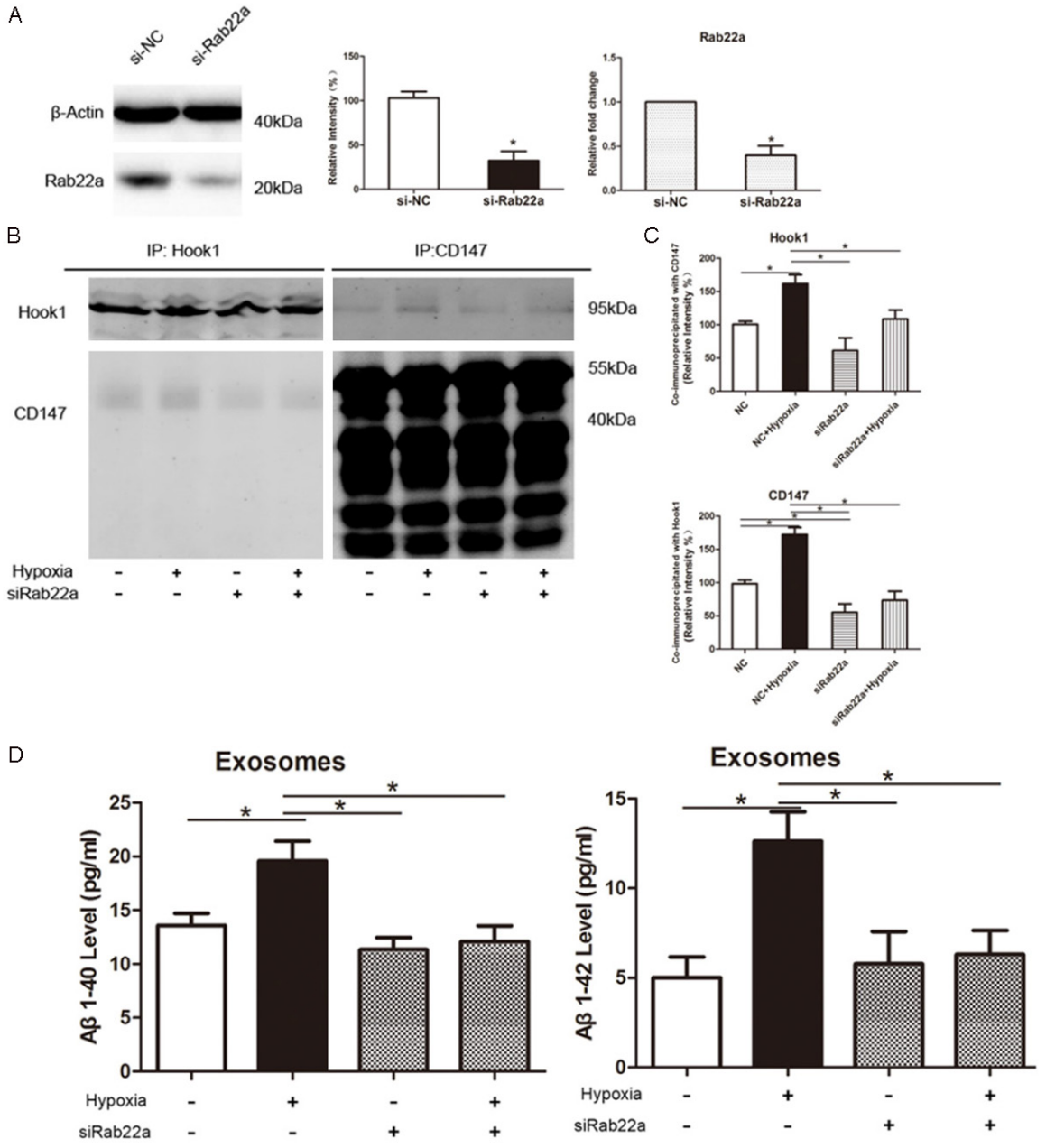

Figure 5. si-Rab22a transfection interferes the interaction between CD147 and Hook1 and decreases Aβ in exosomes under hypoxic condition. A. SH-SY5YAPP^{695} cells were transfected with Rab22a siRNA (si-Rab22a) or negative control (NC). Western blotting and real-time PCR for Rab22a showed the Rab22a expression in cells transfected with Rab22a siRNA reduced significantly. B. Interaction between CD147 and Hook1 was interfered by si-Rab22a. SH-SY5YAPP^{695} cells were transfected with si-Rab22a or negative control, and then exposed to hypoxia for 24 h.

Immunoprecipitation of Hook1 and CD147 was performed to evaluate their interaction. Precipitated immunocomplexes were detected by Western blotting with anti-Hook1 or anti-CD147 antibodies. C. Quantitative densitometric analysis was performed to assess co-IP between CD147 and Hook1 after hypoxia exposure and Rab22a silencing. *P<0.05. D. Aβ40 and Aβ42 were measured in exosomes from hypoxia exposed cells transfected with si-Rab22a by ELISA. Data are expressed as mean ± SEM. *P<0.05.

YAPP^{695} cells. Hook1 and CD147 had red fluorescence (CY3), and green fluorescence (488), respectively. Similarily, hypoxia increased the co-localization of CD147 and Hook1 (**Figure 4D**).

Interfering with the interaction between CD147 and Hook1 reduced hypoxia-induced Aβ accumulation in exosomes

To further investigate whether the interaction between CD147 and Hook1 affects the level of Aβ in exosomes, Rab22a siRNA was used to interfere with the interaction between CD147 and Hook1 because Hook1 works in coordination with microtubules and Rab22a to regulate the recycling of CIE cargo proteins like CD147 back to the PM [17]. Rab22a siRNA (siRab22a) was transfected into SH-SY5YAPP^{695} cells, and the transfection efficiency was verified by Western blotting and real-time PCR (**Figure 5A**). Thereafter, cells were exposed to hypoxia, and the interaction between CD147 and Hook1 and the Aβ production were further investigated. Co-immunoprecipitation assay and Western blotting confirmed the interaction between CD147 and Hook1 decreased after hypoxia exposure in the presence of Rab22a silencing (**Figure 5B**, **5C**). Moreover, Rab22a silencing also inhibited the accumulation of Aβ in exosomes (**Figure 5D**). These findings indicated that interfering with the interaction between CD147 and Hook1 decreases the level of Aβ in exosomes under hypoxic condition.

Hypoxia up-regulated the interaction between CD147 and Hook1 in vivo

The expression of CD147 and Hook1 was detected in the hippocampus of C57BL/6 mice by Western blotting. Results showed that exposure to hypoxia for 5 h significantly increased the CD147 expression although marked increase was not observed in Hook1 expression (**Figure 6A**, **6B**). Co-immunoprecipitation assay was also employed to investigate the interaction between CD147 and Hook1 in the hippocampus of C57BL/6 mice. Endogenous Hook1 and CD147 were immunoprecipitated independently, followed by Western blotting. Results (**Figure 6C**, **6D**) showed the interaction between CD147 and Hook1 increased in the hippocampus of C57BL/6 mice after hypoxia exposure.

Discussion

Exosomes biogenesis involves the inward budding of MVB forming intraluminal vesicles (ILV and exosomes can specifically regulate cell-cell communication as cellular messengers. Exosomes have been found to be related to some neurodegenerative diseases such as A [7, 18, 19]. Aβ production through sequential proteolysis of Amyloid-β (Aβ) precursor protein (APP) by BACE1 and γ-secretase complex has been universally accepted as a factor related to the pathogenesis of AD [20]. There is evidence showing that exposure to hypoxia can augment the amyloidogenic processing of APP, resulting in the accumulation of Aβ peptides [4, 21-23], which might be attributed to the increased activities of β- and γ-secretases in hypoxia. Besides, hypoxia inducible factor-1 α (HIF-1α) protein can directly interact with the γ-secretase complex and increase its activity in a non-transcriptional manner [6]. It has been reported that CD147 expression can be upregulated by hypoxia at both mRNA and protein levels [24]. Our results showed hypoxia increased the levels of Aβ40 and Aβ42 in SH-SY5YAPP^{695} cells derived exosomes, and it significantly increased the CD147 expression *in vitro* and *in vivo*.

CD147 is a type-I immunoglobulin (Ig) transmembrane protein with 269 amino acid (aa) [25, 26] and considered as an integral part of γ-secretase complex, and may influence Aβ accumulation [27]. In this study, cell membrane was isolated from SH-SY5YAPP^{695} cells and the CD147 expression was detected. Results showed that the CD147 expression in cell membrane was significantly increased under hypoxic condition.

APP is a transmembrane protein with a large N-terminal extracellular tail. β-secretase can

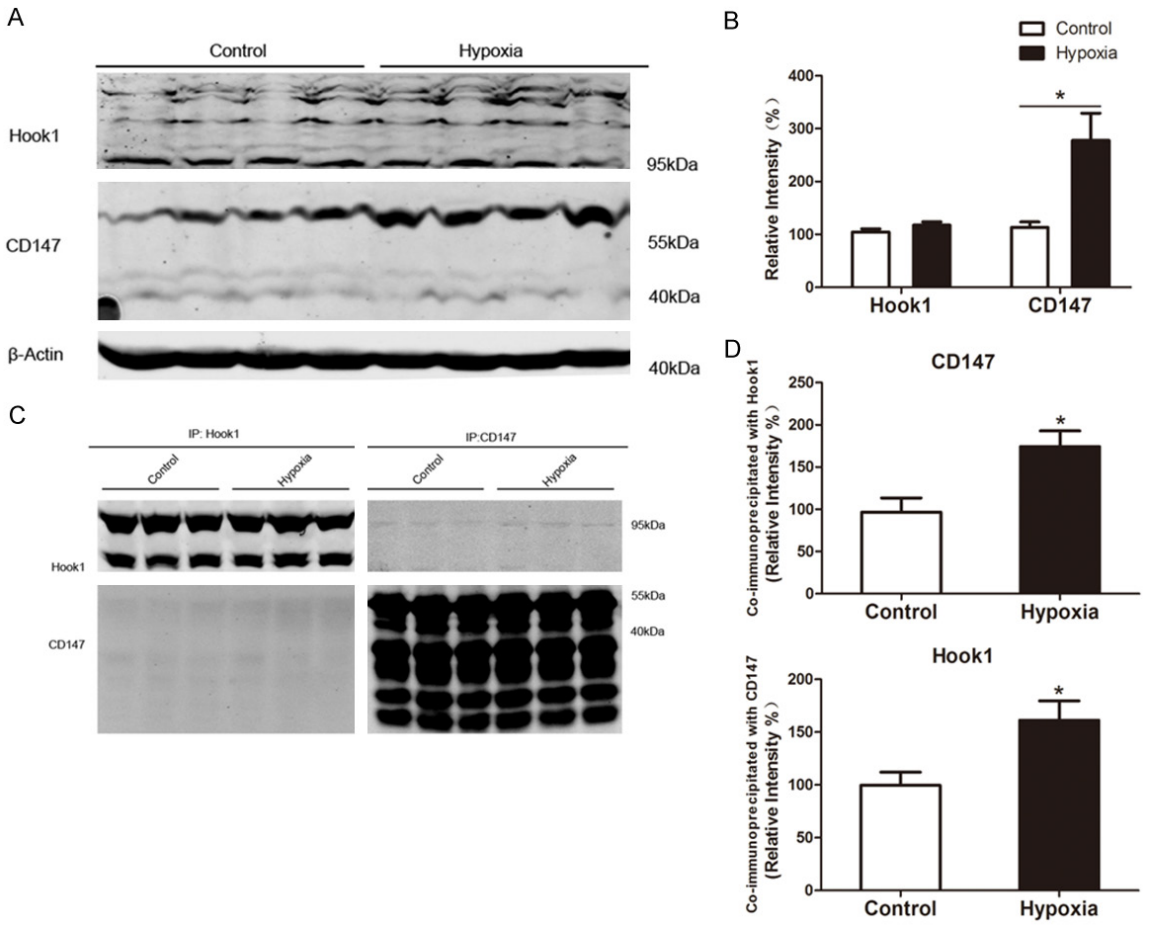

Figure 6. Hypoxia increases the expression of CD147 and the interaction between CD147 and Hook1 in vivo. (A) Hypoxia increased the expression of CD147 in mouse hippocampus. C57bl/6 mice were expose to hypoxia (8% O_2) for 5 h, and then the hippocampus homogenate was prepared for Western blotting (n = 4). (B) Hook1 and CD147 expression (A) was measured by densitometric analysis. (C) Hippocampal homogenates from C57bl/6 mice in both group were subjected to co-IP assay. Then, the immunoprecipitated Hook1 and CD147 were detected by Western blotting to evaluate the interaction between CD147 and Hook1 (n = 3). (D) Quantitative densitometric analysis was performed to assess co-IP between CD147 and Hook1 following hypoxia exposure. Data are expressed as mean ± SEM *P<0.05.

cleave APP just before the Aβ domain, releasing soluble sAPPβ, and the remaining β-CTF (C-terminal fragments), or C99 is cleaved by the γ-secretase complex, releasing the free 40 or 42 amino acid Aβ peptide, but the remaining APP intracellular domain (AICD) is metabolized in the cytoplasm (Blennow et al., 2006). CD147 can regulate the accumulation of Aβ partially though γ-secretase, and γ-secretase is a membrane protein complex that can cleave a variety of type I transmembrane proteins (such as APP) within their transmembranous regions [27]. Therefore, we speculate that APP may directly interact with CD147. In this study, co-immunoprecipitation assay was employed to investigate the direct interaction between APP and CD147 in SH-SY5YAPP^{695} cells. Our findings indicated that CD147 was able to regulate the accumulation of Aβ, which might be partially attributed to the interaction between CD147 and APP.

To investigate the impact of the interaction between CD147 and Hook1 on the Aβ accumulation in exosomes, Rab22a siRNA was transfected into SH-SY5YAPP^{695} cells to interfere with the interaction between CD147 and Hook1. The small GTPase Rab22a has been found to act on the endocytic pathway at multiple levels. It may significantly affect the function of endosomes and regulate the recycling of cargoes trafficking [28, 29]. It has been

reported that Hook1, microtubules, and Rab-22a work in coordination to directly recycle the cargo [17]. Maldonado-Baez et al. found overexpression of Hook1 rescued the Rab22a S19N dominant negative phenotype, overexpression of Rab22a rescued the Hook1 dominant negative effect by restoring the endosomal sorting of CIE cargo, and the constitutively active Rab22a also rescued the Hook1 dominant-negative phenotype, but Rab22a S19N (a negative mutant, with reduced affinity to GTP) had not this effect. The reciprocal rescue of their respective dominant negative phenotypes and their co-localization on membranes suggest that they work at the same step to control the direct recycling of CIE cargo proteins from early endosome [17]. In present study, our results showed that Rab22a siRNA decreased the interaction between CD147 and Hook1 (**Figure 5B**, **5C**), and the level of Aβ in exosomes under hypoxic condition reduced to the level in control group (**Figure 5D**). These results indicated that si-Rab22a eliminated the Aβ accumulation in exosomes after hypoxia exposure.

Previous studies have shown that Aβ could be packaged in MVBs and exosomes and shed to the extracellular microenvironment [8]. Aβ in exosomes has been reported to be associated with the pathogenesis of AD, particularly the extracellular amyloid fibril formation [30]. It was also shown that the spreading of AD lesions might be related to the endosomes/MVBs and exosomes in a mode of prion-like propagation [11]. Exosomes provide an explanation for the shipping of Aβ from extracranial tissues to the brain, and contribute to amyloid deposition [10]. β-CTF has been found to be trafficked to various endosomal compartments, including MVBs [31], and the full-length APP, APPCTFs, and enzymes such as BACE1, presenilin 1 (PS1), PS2, nicastrin (components of γ-secretase complex) were also identified in the exosomes [32, 33]. Moreover, the inhibition of γ-secretase increased APP-CTFs in exosomes. Thus, there might be an unidentified site of APP cleavage [33]. Unfortunately, it still remains undefined whether the cleavage occurs in these exosomes or prior to their being packaged in exosomes. The release of Aβ peptides from the intracellular space leads to the accumulation of extracellular Aβ, but how the released extracellular Aβ peptides is generated in the intracellular space is still unclear. It seems that a majority of Aβ40 and Aβ42 are produced during the internalization and endosomal processing of APP in endosomes [34, 35]. There are conflicting findings about whether Aβ is generated early in the secretory trafficking of APP such as in endoplasmic reticulum, intermediate compartment, and early Golgi, or mainly after APP reaches the cell surface [36].

Taken together, our findings suggest that hypoxia is able to increase Aβ in exosomes by enhancing the interaction between CD147 and Hook1, although the exosomal Aβ which is up-regulated after hypoxia is only a small part of the entire Aβ peptides. Since Aβ peptides in exosomes can promote their spread in the extracellular space and may further contribute to the progress of neurodegeneration, our results might provide a novel insight for the pathogenesis of AD.

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Disclosure of conflict of interest

None.

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