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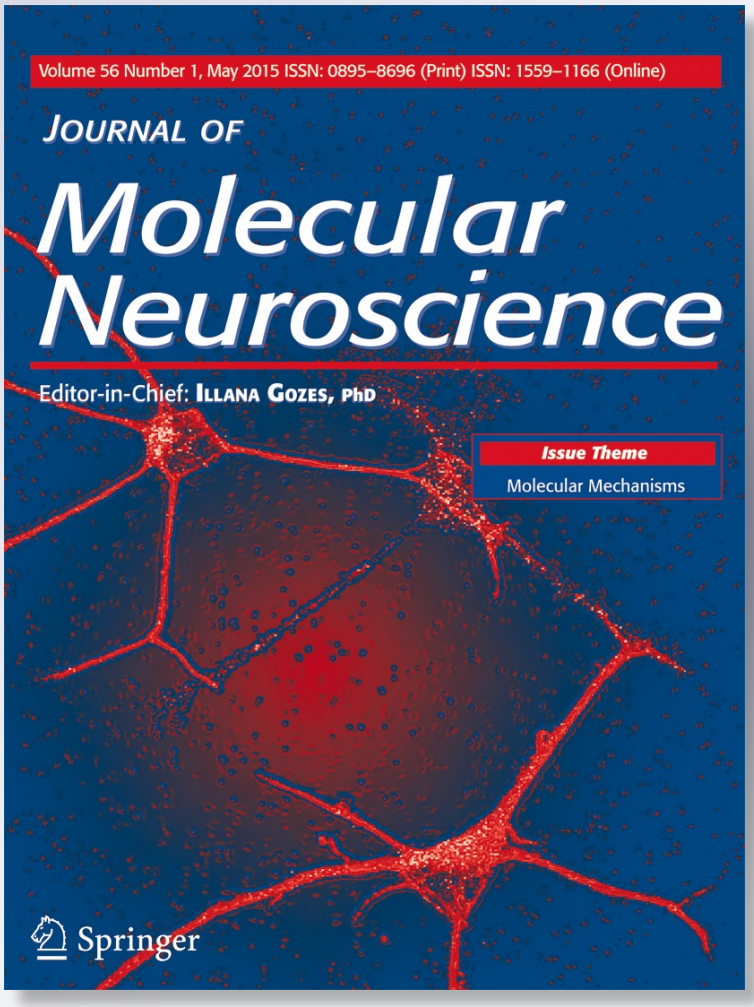

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Haptoglobin Modulates Beta-Amyloid Uptake by U-87 MG Astrocyte Cell Line

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Abstract Accumulation of beta-amyloid (Aβ) in the extracellular space, which is one of the hallmarks of Alzheimer's disease (AD), depends on the balance between its synthesis and clearance. The physiological role of extracellular chaperones, capable of affecting early events in the amyloid cascade, is increasingly being investigated by many research groups. Among these proteins, we focused on haptoglobin, which we recently found to form a complex with beta-amyloid in brain tissues or cerebrospinal fluids from patients with AD. We also previously reported that haptoglobin increases with age in rat hippocampus. Major aim of this study was to evaluate whether haptoglobin influences Aβ interaction with astrocytes and its internalization into these cells. Haptoglobin effect on Aβ-induced cell death was also explored. We report here that haptoglobin impairs Aβ uptake by human glioblastoma–astrocytoma cell line U-87 MG and limits the toxicity of this peptide on these cells. Of note, our data also show that Aβ can stimulate haptoglobin release by astrocyte cell lines. The study of the risk of developing AD should be focused not only on the analysis of Aβ but also on the level of critical ligands, such as haptoglobin, able to influence peptide aggregation or clearance.

Keywords Haptoglobin · Beta-amyloid · Astrocyte · Brain · Clearance · Alzheimer's disease

Introduction

Alzheimer's disease (AD) is the most common form of age-related dementia in the elderly. Major neuropathological and neurochemical hallmarks of AD include the extracellular accumulation of amyloid-β peptide (Aβ) in brain senile plaques and the intracellular formation of neurofibrillary tangles composed of hyperphosphorylated Tau protein (Huang and Jiang 2009). Aggregation of Aβ into oligomers and fibrils was hypothesized to lead to a pathological cascade resulting in synaptic dysfunction, neuronal loss, and, ultimately, cognitive decline (Holtzman et al. 2011). Accumulation of Aβ in brain senile plaques results from the imbalance between production and clearance of Aβ (Janciauskiene et al. 1998). Therefore, a better characterization of the mechanisms underlying Aβ elimination from the brain may lead to insights into the pathogenesis of the disease and reveal novel therapeutic targets. In fact, Aβ clearance from the central nervous system (CNS), not only its production, was shown to be impaired in individuals with late-onset AD (Mawuenyega et al. 2010). Several clearance pathways for Aβ exist in the CNS, including cellular uptake and lysosomal degradation, transport across the blood–brain barrier (BBB), extracellular degradation by proteolytic enzymes, and bulk flow drainage of interstitial fluid and cerebrospinal fluid (CSF) (Bell et al. 2007; Deane et al. 2008; Basak et al. 2012; Castellano et al. 2012; Iliff et al. 2012). Although current evidence suggests that astrocytes might play a protective role in AD by shielding neurons from the toxic effects of extracellular senile plaques (Guénette 2003), the exact role of these cells in amyloid clearance needs further investigations. Recently, it has been reported that activated astrocytes can internalize and degrade

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Aβ (Verghese et al. 2013; Li et al. 2014; Mulder et al. 2014) probably in the attempt to reduce Aβ exposure to neurons. Next to Aβ, several other proteins called amyloid-associated proteins (AAPs), such as serum amyloid P, complement factor C1q, alpha1-antichymotrypsin, haptoglobin (Hpt), apolipoprotein J (ApoJ), and apolipoprotein E (ApoE) are known to co-localize with Aβ in the amyloid plaque (Veerhuis et al. 2003; Yerbury et al. 2005). Due to their proximity with senile plaques and to their capacity to interact with Aβ, AAPs may be involved in AD pathogenesis. The acute phase protein Hpt is mainly synthesized in the liver and is so far known for its role in hemoglobin binding and transport to the liver (Quaye 2008). Hpt was initially identified as a marker of BBB dysfunction (Chamoun et al. 2001). Further, some studies pointed out that this protein may be produced in the brain in response to different stress stimuli (Lee et al. 2002; Borsody et al. 2006; Zhao et al. 2009), and increased level of Hpt was found in CSF from patients with AD (Johnson et al. 1992; Yerbury and Wilson 2010) or other neurodegenerative diseases such as Parkinson' and Huntington's disease (Argüelles et al. 2010; Huang et al. 2011). Hpt was reported to co-localize with amyloid plaques in AD (Powers et al. 1981), to bind Aβ and inhibit Aβ fibril formation in vitro (Wilson et al. 2008; Yerbury et al. 2009). Furthermore, we recently provided evidence that Hpt modulates ApoE-mediated cholesterol trafficking in neuroblastoma cell lines and that its concentration increases with the age in rat hippocampus as well as in human CSF (Spagnuolo et al. 2014a). The ability of Hpt to bind Aβ raises the possibility that this protein might influence Aβ homeostasis and/or Aβ interaction with brain cells. The aim of this study was to evaluate whether Hpt influences Aβ interaction with astrocytes and its internalization. Since Aβ accumulation has been reported as a key event in Aβ-mediated cell degeneration (Verbeek et al. 1997), Hpt effect on Aβ-induced cell death was also explored.

Materials and Methods

Materials

Bovine serum albumin (BSA) fraction V, gelatin, rabbit anti-human Hpt IgG, mouse anti-β actin IgG, goat anti-rabbit horseradish peroxidase-conjugated IgG (GAR-HRP), goat anti-mouse horseradish peroxidase-conjugated IgG (GAM-HRP), Crystal Violet, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), were purchased from Sigma-Aldrich (St. Louis, MO, USA). The dye reagent for protein titration, enhanced chemiluminescence (ECL) reagents, and the polyvinylidene difluoride (PVDF) membrane were from Bio-Rad (Bio-Rad, Hercules, CA, USA). Polystyrene 96-well enzyme-linked immunosorbent assay (ELISA) MaxiSorp plates were purchased from Nunc (Roskilde, Denmark). Recombinant human ApoE3 was from PeproTech (London, UK). Kodak Biomax light film, Sephacryl S-200, CNBr-activated Sepharose 4 Fast Flow, and Blue Sepharose 6 Fast Flow resins were from GE-Healthcare Life Sciences (Milan, Italy). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were from BioWhittaker (Verseviers, Belgium). L-Glutamine, TrypLE Express, penicillin, and streptomycin were from Gibco (Life Technologies Italy, Monza, Italy). Cell culture flasks (25 cm^2), 96-well cell culture plates, 24-well culture plates, and sterile pipettes of Beckton-Dickinson (Milan, Italy) were used.

Purification of Hpt

Hpt was isolated from plasma of healthy subjects (phenotype 1–1) by a multi-step purification procedure as previously reported (Cigliano et al. 2009). Hpt was over 98 % pure, as assessed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and densitometric analysis of Coomassie-stained bands. The molarity of purified Hpt was determined by measuring the protein concentration (Bradford 1976) and calculating the molecular weight of the monomer αβ as previously described (Cigliano et al. 2003).

Aβ Preparation

Human $Aβ_{1–42}$ (GL Biochem, Shanghai; purity>95 % as assessed by HPLC) was produced by chemical synthesis. In order to obtain a solution free of aggregates and fibrils, the lyophilized peptide was treated with 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) (Stine et al. 2003). After HFIP evaporation under nitrogen stream, the peptide was resuspended in anhydrous dimethyl sulfoxide, sonicated for 20 min (bath sonicator model 3200MH; Soltec), and its concentration was verified by a colorimetric assay (Bradford 1976) using insulin as standard for calibration.

Cell Culture

The human glioblastoma–astrocytoma cell line U-87 MG was kindly provided by the Institute of Genetics and Biophysics (CNR, Naples, Italy). U-87 MG cells express the astrocyte cell marker glial fibrillary acidic protein and are widely used as astrocyte model in vitro (Bertrand et al. 2009; Chen et al. 2010; Moser and Fritzler 2010; Li et al. 2014). Cells (400,000) were seeded in T-25 tissue culture flasks (25 cm^2 surface) and grown in DMEM supplemented with 10 % FBS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (complete medium) at 37 °C, under humidified atmosphere of 5 % CO_2 in air. The medium was changed twice a week, and cells were sub-cultivated when confluent.

Aβ Binding to U-87MG by ELISA

Aβ ability to bind U-87 MG cell line was evaluated by ELISA. Cells were seeded into a 96-well plate (15,000 cells/well) and cultured for 20 h in complete medium. Then, the cells were washed with phosphate-buffered saline (PBS) and fixed by incubation (30 min, 4 °C) with 0.3 % glutaraldheyde in PBS. After washing with PBS, the wells were blocked with PBS containing 1 % BSA (overnight, 4 °C), and then incubated (2 h, 37 °C) with different amounts of $A\beta_{1–42}$ (0.5, 1, 2.5, 5, 10, or 20 μM in PBS). The amount of Aβ bound to cells was measured by treatment with 50 μl of mouse anti-Aβ IgG 6E10 (1:2,000 dilution in PBS; 1 h, 37 °C), followed by 50 μl of GAM-HRP IgG (1:6,000 dilution; 1 h, 37 °C) and color development at 492 nm. Absorbance values were converted to the percent of the value obtained in the presence of the higher amount of Aβ used in the assay (assumed as 100 % of binding). Data were analyzed by a nonlinear regression fit algorithm (GraphPad Prism v 5.01) for calculating the equilibrium binding constant (Kd).

In order to evaluate the effect of Hpt on Aβ binding to cells, U-87 MG, after blocking, were incubated (2 h, 37 °C) with aliquots (55 μl) from mixtures containing 4 μM $A\beta_{1–42}$ and different concentrations of Hpt (0, 0.04, 0.08, 0.4, 1.2, 3, or 6 μM). The amount of $A\beta_{1–42}$ bound to cells was measured by incubation with mouse anti-Aβ IgG 6E10 followed by GAM-HRP IgG as above-described. Absorbance values were converted to the percent of the value obtained in the absence of Hpt.

Aβ Internalization into Astrocytes Cell Line

U-87 MG were cultured (96-well plates; 15,000 cells/well) in complete medium for 20 h. After medium removal, cells were rinsed with DMEM and incubated (1, 1.5, 3, 6, or 20 h, 37 °C) in DMEM containing 5 μM $A\beta_{1–42}$. At each time point, media samples were collected and cleared of any cellular debris by centrifugation (400×*g*, 5 min). The cells were extensively washed with DMEM, detached by treatment (5 min) with 50 μl of trypsin (TrypLE Express, Gibco), which also removes Aβ bound to cell membrane (Verghese et al. 2013), lysed with RIPA buffer (150 mM NaCl, 50 mM Tris–HCl, 1 % NP-40, 0.5 % sodium deoxycholate, pH 8) containing Tissue Protease Inhibitor Cocktail (Sigma-Aldrich, 1:200, *v/v*), and finally centrifuged (12,000×*g*, 30 min). Protein concentration in cell lysates was then measured (Bradford 1976). Aliquots of media samples and cell lysates were analyzed by electrophoresis on 16 % polyacrylamide gel, under denaturing but non-reducing conditions followed by Western blotting, and by ELISA for measuring Aβ concentration, as described below.

The effect of Hpt on Aβ uptake by astrocytes was evaluated by incubating (1.5, 3, 6, or 20 h, 37 °C) U-87 MG in DMEM containing 5 μM $A\beta_{1–42}$ in the absence or presence of 1.6 μM Hpt. At each time point, cell culture supernatants and cell lysates were collected and then analyzed by electrophoresis on 4–20 % polyacrylamide gel, under denaturing but non-reducing conditions (4–20 PAGE-D) followed by Western blotting, and by ELISA.

In order to compare Hpt and ApoE effect on Aβ uptake by astrocytes, U-87 MG were incubated (1.5, 3, 6, or 20 h, 37 °C) in DMEM containing 5 μM $A\beta_{1–42}$ and 1.6 μM Hpt, or 1.6 μM ApoE, or 1.6 μM Hpt and 1.6 μM ApoE. At each time point, cell culture supernatants and lysates were collected and analyzed by both Western blotting and ELISA. The experiment was carried out using liposome-embedded ApoE, as this apolipoprotein, in brain, is mostly associated with HDL-like particles (Vance and Hayashi 2010). Liposomes containing ApoE3 (ApoE/lecithin=1:133 molar contribution, namely LipoE3) were prepared by the cholate dialysis method (Chen and Albers 1982; Spagnuolo et al. 2014b).

The dose–effect of Hpt was assessed by incubating the cells (3 h, 37 °C) in DMEM containing 5 μM $A\beta_{1–42}$ and different amounts of Hpt (0, 0.5, 1.6, or 3 μM). At the end of incubation, media samples and cell lysates were analyzed by Western blotting and ELISA.

Electrophoresis and Western Blotting

Aliquots of supernatants (20 μl), collected from U-87 MG incubated with $A\beta_{1–42}$ in the absence or presence of Hpt, were treated with 3× O'Farrell buffer without β-mercaptoethanol (Spagnuolo et al. 2014b) and then analyzed by electrophoresis (on 16 % or on 4–20 % polyacrylamide gel) under denaturing but non-reducing conditions, followed by Western blotting. After proteins transfer onto PVDF membrane (1 h, under electric field), the membrane was rinsed in T-TBS (130 mM NaCl, 20 mM Tris–HCl, 0.05 % Tween 20, pH 7.4) and blocked with T-TBS containing 5 % non-fat milk (overnight, 4 °C). The membrane was then incubated (1 h, 37 °C) with mouse anti-Aβ IgG 6E10 (1:4000 dilution in T-TBS containing 0.25 % non-fat milk), followed by GAM-HRP IgG (1:20,000 dilution; 1 h, 37 °C), for revealing antigens containing Aβ epitopes. The immunocomplexes were detected by the ECL detection system. The influence of Hpt on the formation of Aβ aggregates was investigated by incubating (0–20 h, 37 °C) 5 μM $A\beta_{1–42}$ in DMEM without cells, in the absence or presence of 1.6 μM Hpt. Aliquots of media samples were then analyzed 4–20 PAGE-D followed by Western blotting.

Samples (8 μg) of U-87 MG lysates were analyzed by 4–20 % PAGE-D and immunoblotting, essentially as described above for supernatants. In particular, Aβ was revealed by incubation (overnight at 4 °C) with mouse anti-Aβ IgG 6E10 (1:500 dilution), followed by GAM-HRP IgG (1:1500 dilution; 1 h, 37 °C), and ECL staining. In some experiments, after Aβ detection, the membrane was extensively washed

with T-TBS and submerged in stripping buffer (0.4 M NaOH, 45 min, room temperature) for reprobing with anti-β-actin. β-Actin was revealed by incubation with mouse anti-β-actin IgG (1:500 dilution; 1 h, 37 °C), followed by GAM-HRP IgG (1:2000 dilution; 1 h, 37 °C), and ECL staining. Quantitative densitometry was then carried out by analyzing the digital images of membranes by the Gel-Pro Analyzer software (Media Cybernetics, Silver Spring, MA, USA). Band intensities were calculated as integrated optical density (IOD).

Enzyme-Linked Immunosorbent Assay Aβ concentration in supernatants and cell lysates of U-87 MG was measured by ELISA. Samples were diluted (supernatants, 1:1000, 1:3000, and 1:6000; cell lysates, 1:100, 1:300, and 1:1000) with coating buffer (7 mM Na_2CO_3, 17 mM $NaHCO_3$, and 1.5 mM NaN_3, pH 9.6) and incubated in the wells of a microtiter plate (overnight, 4 °C). The assay was performed as previously published (Cigliano et al. 2009) by using mouse anti-Aβ IgG 6E10 (1:500 dilution in T-TBS containing 0.25 % BSA) followed by 50 μl of GAM-HRP IgG (1:6000 dilution).

Analysis of Aβ Effect on Cell Survival

U-87 MG were seeded into 96-well plate (5000 cells/well) and cultured for 20 h in complete medium. Cells were then rinsed with DMEM and incubated (24 h, 37 °C) in DMEM containing different amounts of $Aβ_{1–42}$ (0, 0.5, 1.5, 3, 6, 10, or 15 μM). Cell survival was evaluated by MTT reduction assay, by incubating the wells with 100 μl of MTT (0.5 mg/ml in DMEM without Phenol Red; 3 h, 37 °C). After incubation, 100 μl of a solution containing 0.1 M HCl in isopropanol was added to each well. Absorbance at 595 nm was then measured. The data were expressed as percentage of cell survival, assuming the absorbance value from cells cultured in the absence of Aβ as 100 %.

The effect of Hpt was evaluated by incubating (24 h, 37 °C) the cells in DMEM containing $Aβ_{1–42}$ (6 μM) and different amounts of Hpt (0, 0.15, 0.3, 0.6, or 1.2 μM). Cell survival was then measured by MTT assay, and by Crystal Violet assay, and data were expressed as cell survival percentage.

Hpt Synthesis and Secretion by U-87 MG Cell Line

U-87 MG were seeded into 24-well plate (100,000 cells/well) in complete medium and cultured for 20 h. After medium removal, cells were rinsed with DMEM and incubated (10 h, 37 °C) in DMEM containing different amounts of $Aβ_{1–42}$ (0, 3, or 6 μM). Media samples were collected, centrifuged (400×*g*, 5 min), and finally concentrated 7-fold with centrifugal evaporator. Cells were extensively washed with DMEM, detached by treatment with trypsin, washed with ice-cold PBS, and finally lysed with RIPA buffer (supplemented with Tissue Protease Inhibitor Cocktail, 1:200, *v*/*v*). Aliquots of cell culture supernatants (40 μl) or lysates (28 μg) were fractionated by electrophoresis on 15 % polyacrylamide gel, under denaturing and reducing conditions (Spagnuolo et al. 2014b). After protein transfer onto PVDF membrane, Hpt was revealed by incubation (1 h, 37 °C) with rabbit anti-human Hpt IgG (1:500 dilution), followed by GAR-HRP IgG (1:3000 dilution; 1 h, 37 °C) and ECL staining.

For cell lysates analysis, the membrane was extensively washed with T-TBS, after Hpt detection, and submerged in stripping buffer for reprobing with anti-β-actin. After washing with T-TBS, the membrane was incubated (1 h, 37 °C) with mouse anti-β-actin IgG (1:500 dilution), followed by GAM-HRP IgG (1:2000 dilution; 1 h, 37 °C). The immunocomplexes were detected by the ECL detection system, and densitometric analysis of the signal was carried out.

Statistical Analysis In all experiments, samples were processed in triplicate, and data were expressed as mean value± SEM. The program "GraphPad Prism 5.01" (GraphPad Software, San Diego, CA, USA) was used to perform regression analysis, Student's *t* test, for comparing two groups of data, and one-way ANOVA, followed by Tukey's test, for multiple group comparisons. $P<0.05$ was set as indicating significance.

Results

Hpt Influence on Aβ Binding to Astrocytes

Aβ binding and uptake by primary astrocytes, as well as by U-87 MG cell line, was previously described (Wyss-Coray et al. 2003; Nielsen et al. 2009, 2010; Li et al. 2014), and both the low-density lipoprotein receptor (LDLR) and the LDLR-related protein-1 (LRP-1) were reported to act as Aβ receptors (Nagele et al. 2003; Fuentealba et al. 2010; Basak et al. 2012; Kanekiyo et al. 2012). Further, it was recently published that LRP1 is involved in Aβ internalization in U-87 MG cells (Li et al. 2014). A preliminary cell-based ELISA was performed to evaluate the efficiency of Aβ binding to U-87 MG, in our experimental conditions. To this aim, the cells, after glutaraldheyde fixation, were incubated with different amounts of $Aβ_{1–42}$ (0.5–20 μM). As shown in Fig. 1a, Aβ was able to bind astrocytes at any concentration assayed. The amount of peptide bound to cells increased with the concentration of Aβ in the incubation medium ($p<0.01$), and the binding plateau was reached at 10 μM. The Kd of Aβ binding to astrocyte, calculated from nonlinear regression fit, was 1.761±0.289 μM.

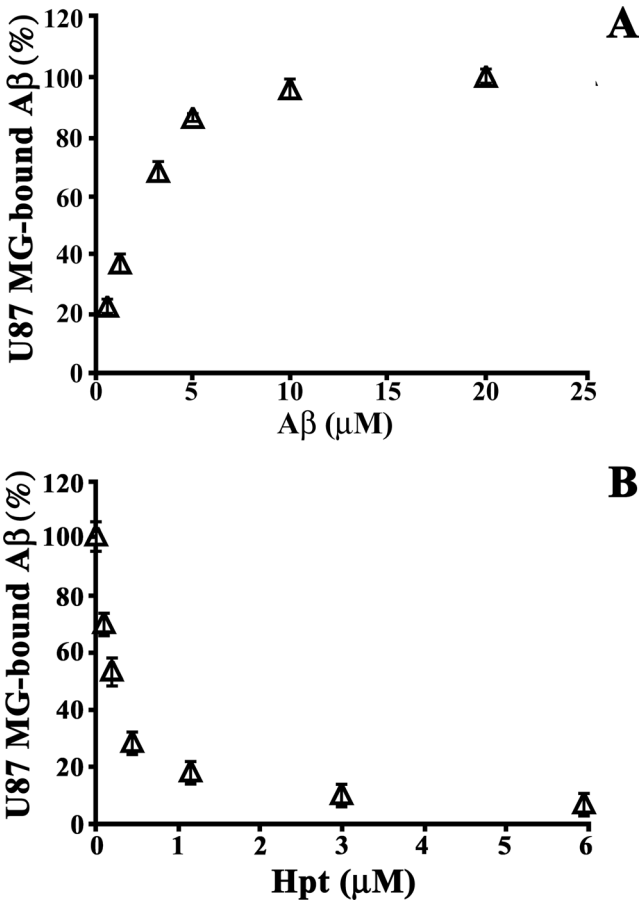

Fig. 1 Aβ binding to astrocytes. **a** U-87 MG (15,000 cells/well) were fixed into the wells of a 96-well plate and then incubated with different concentrations (0.5–20 μM) of $Aβ_{1–42}$. The amount of Aβ bound to cells was detected by mouse anti-Aβ IgG 6E10 IgG and GAM-HRP, measured as absorbance at 492 nm, and reported as percentage of the value obtained with 20 μM $Aβ_{1–42}$ (assumed as 100 % of Aβ binding to cells). Samples were processed in triplicate. Data were expressed as mean±SEM versus μmolar concentration. One representative of at least three different experiments is represented. **b** U-87 MG (15,000 cells/well) were fixed into the wells of a 96-well plate and then incubated with mixtures containing 4 μM $Aβ_{1–42}$ and different amounts of Hpt (0–6 μM). The amount of Aβ bound to cells was measured by incubation with mouse anti-Aβ IgG 6E10 IgG, followed by GAM-HRP and color development at 492 nm. Data are reported as percent of the value obtained by incubation in the absence of Hpt (assumed as 100 % of Aβ binding), and expressed as mean±SEM. One representative of at least three different experiments is represented

As Aβ interaction with cell surface is crucial for peptide uptake and intracellular degradation (Paresce et al. 1996; Nagele et al. 2003; Wilhelmus et al. 2007), we investigated whether Hpt, due to its interaction with Aβ, influences the binding of this peptide to U-87 MG astrocyte line. In order to assess whether Hpt affects Aβ binding to astrocytes, the cells were incubated with mixtures containing 4 μM $Aβ_{1–42}$ and different amounts of Hpt (0–6 μM, corresponding to 0–1.5 molar excess over the concentration of Aβ). As shown in Fig. 1b, Aβ binding to the fixed cells significantly decreased ($p<0.01$) as the amount of Hpt in the incubation mixture increased. In particular, Aβ binding to astrocytes was reduced of 30 % ($p=0.003$) by 0.04 μM Hpt and of 48 % ($p=0.002$) by 0.08 μM Hpt (a concentration about 50-fold lower than Aβ in the mixture). Further, the binding dropped down to 9 and 6 % in presence of 3 and 6 μM Hpt, respectively ($p<0.001$). The concentration of Hpt producing half-maximal inhibition (IC50) of Aβ binding to astrocyte, calculated from nonlinear regression fit, was 80.5 nM.

Altogether, our results, although concerning cells fixed with glutaraldheyde, which might affect their surface properties, suggest that Hpt strongly interferes with Aβ binding to astrocyte cell line, likely by displacing the peptide from its cell receptor and/or by reducing peptide aggregates formation, and this effect is more pronounced, when the Hpt/Aβ ratio increases.

U-87 MG Cell Line Takes up and Degrades Soluble $Aβ_{1–42}$

In order to investigate the contribution of astrocytes to the clearance of soluble Aβ, U-87 MG cells were incubated with 5 μM $Aβ_{1–42}$ for different times (1–20 h). Aliquots of supernatants and cell lysates, collected at each time point, were then analyzed by Western blotting and by ELISA for revealing and titrating $Aβ_{1–42}$. Western blotting analysis revealed that soluble $Aβ_{1–42}$ in the cell culture supernatant decreased in a time-dependent manner (Fig. 2a), thus showing that this cell line is able to clear $Aβ_{1–42}$ from extracellular milieu. The peptide concentration, assessed by ELISA, was found to linearly decrease from 1 to 6 h of incubation with cells (Fig. 2b; $r=-0.97$; $p=0.04$). In particular, $Aβ_{1–42}$ level was reduced of about 43 % ($p<0.001$) within 3 h and of 92 % after 20 h. The analysis of cell lysates revealed that intracellular $Aβ_{1–42}$ increased in a time-dependent manner (Fig. 2c), thus confirming that U-87 MG cell line is able to uptake Aβ from the extracellular compartment. In particular, the concentration of internalized peptide increased linearly with time from 1 to 6 h (Fig. 2d; $r=0.93$; $p=0.02$), raising about 5-fold ($p<0.001$).

Hpt Influence on Astrocyte-Mediated $Aβ_{1–42}$ Clearance

As our results suggest that Hpt might interfere with $Aβ_{1–42}$ binding to astrocytes, we investigated whether Hpt affects the uptake of this peptide. To this aim, U-87 MG were incubated with 5 μM $Aβ_{1–42}$, in the absence or presence of 1.6 μM Hpt, for different times (1.5–20 h). Aliquots of supernatants and cell lysates were then analyzed both by immunoblotting and by ELISA for measuring, at each time point, the amount of $Aβ_{1–42}$. Western blotting analysis demonstrated a significant time-dependent decrease in soluble $Aβ_{1–42}$ in the cell culture supernatant (Fig. 3a), which was affected by Hpt. Indeed, a higher amount of $Aβ_{1–42}$ was detected in the supernatants collected from cells incubated with 1.6 μM Hpt for 1.5, 3, or

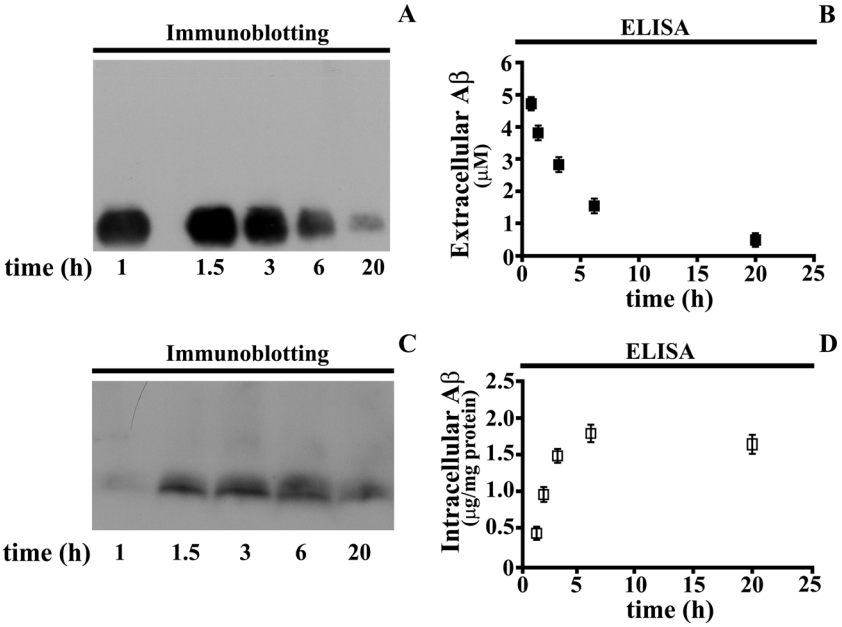

Fig. 2 $A\beta_{1–42}$ uptake by U-87 MG cell line. U-87 MG (96-well plates; 15,000 cells/well) were incubated (1, 1.5, 3, 6, or 20 h, 37 °C) in DMEM containing 5 μM $A\beta_{1–42}$. At each time point, media samples and cell lysates were collected and analyzed by Western blotting (**a**, **c**), and by ELISA (**b**, **d**) for measuring Aβ concentration. **a** Representative Western blot of cell culture supernatants. Samples were fractionated by 16 % SDS-PAGE, under denaturing but non reducing conditions followed by Western blotting. Immunocomplexes were detected by mouse anti-Aβ IgG 6E10 IgG and GAM-HRP. **b** Representative ELISA titration of Aβ in cell culture supernatants. Aβ concentration, in cell culture supernatants collected at each time point, was measured by mouse anti-Aβ IgG 6E10 IgG, followed by GAM-HRP, and color development at 492 nm. The calibration curve was obtained by assaying the immunoreactivity of 0.05–0.1-0.25–0.50–0.75–1.0–2.0 ng of commercial Aβ standard. Samples were processed in triplicate. Data were expressed as mean±SEM. **c** Representative Western blot of cell lysates. Samples were fractionated by 16 % SDS-PAGE, under denaturing but non reducing conditions followed by Western blotting. Immunocomplexes were detected by mouse anti-Aβ IgG 6E10 IgG and GAM-HRP. **d** Representative ELISA titration of intracellular Aβ. Aβ concentration, in cell lysates collected at each time point, was measured by mouse anti-Aβ IgG 6E10 IgG, followed by GAM-HRP, and colour development at 492 nm. The calibration curve was obtained by assaying the immunoreactivity of 0.05–0.1−0.25–0.50 −0.75–1.0–2.0 ng of commercial Aβ standard. Samples were processed in triplicate. Data were reported as microgram of peptide per microgram of protein, and expressed as mean±SEM. For each panel, one representative of at least three different experiments is represented

6 h compared to those from cells incubated in the absence of Hpt, thus suggesting that, within this time interval, the removal of $A\beta_{1–42}$ from the culture medium is influenced by the presence of Hpt. Accordingly, the peptide concentration, assessed by ELISA, was higher (1.6±0.08-fold, $p<0.001$) in the supernatant collected after 1.5, 3, or 6 h of incubation with $A\beta_{1–42}$ and Hpt than in that from cells incubated with $A\beta_{1–42}$ alone (Fig. 3b). In particular, the level of $A\beta_{1–42}$ was reduced of about 34 % ($p=0.01$) within 1.5 h in the absence of Hpt, while it was unchanged, compared to time zero, in the presence of Hpt. Further $A\beta_{1–42}$ concentration linearly decreased from 1.5 to 6 h of incubation in the absence of Hpt (Fig. 3b; $r=-0.95$, $p=0.04$), and from 1.5 to 20 h of incubation in the presence of Hpt (Fig. 3b, $r=-0.97$, $p=0.03$). The analysis of cell lysates by immunoblotting showed a time-dependent increase in intracellular $A\beta_{1–42}$ (Fig. 3c), which was affected by the presence of Hpt. Indeed, the amount of peptide internalized raised until 6 h, when cells were incubated with peptide alone (Fig. 3c), and until 3 h when cells were incubated in the presence of Hpt. In particular, ELISA revealed that the concentration of internalized peptide increased (about 2-fold; $P<0.001$) after 6 h of incubation in the absence of Hpt compared to 1.5 h, but did not further change after 20 h (Fig. 3d). Conversely, when cells were incubated with Hpt, a mild increase in the level of internalized peptide (about 1.2-fold; $P=0.02$) was detected after 3 h when compared to 1.5 h, and no change was found after 6 h. Further, the concentration of peptide was significantly reduced (about 1.3-fold, $P=0.02$; Fig. 3d) after 20 h of incubation in comparison to 3 h. These results demonstrate that Hpt interferes with $A\beta_{1–42}$ internalization by astrocytes. In order to examine the aggregation state of Aβ, in absence or in the presence of Hpt, a control experiment was performed, in the same experimental conditions, by incubating (0–20 h) 5 μM $A\beta_{1–42}$ in DMEM without cells. As shown in Figure S1, peptide aggregates were not clearly detected in mixtures incubated for 1.5–6 h, both in the absence and presence of Hpt. Conversely, in the mixture incubated 20 h without Hpt, a smear of $A\beta_{1–42}$ aggregates was revealed, and this aggregate formation was highly limited in the presence of Hpt, thus confirming that Hpt contributes to inhibit peptide aggregation.

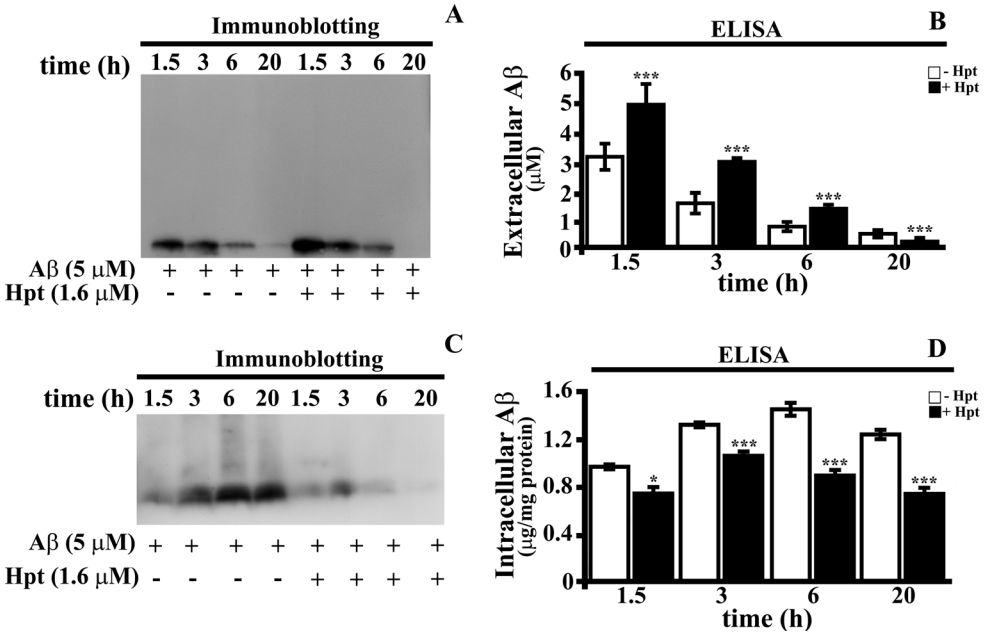

Fig. 3 Hpt influence on astrocyte-mediated $A\beta_{1–42}$ clearance. U-87 MG (96-well plates; 15,000 cells/well) were incubated (1.5, 3, 6, or 20 h, 37 °C) in DMEM containing 5 μM $A\beta_{1–42}$, in the absence or presence of 1.6 μM Hpt. At each time point, media samples and cell lysates were collected and analyzed by Western blotting (**a**, **c**) and by ELISA (**b**, **d**) for measuring Aβ concentration. **a** Representative Western blot of cell culture supernatants. Samples were fractionated by 4–20 % SDS-PAGE, under denaturing but non-reducing conditions followed by Western blotting. Immunocomplexes were detected by mouse anti-Aβ IgG 6E10 IgG and GAM-HRP. **b** Representative ELISA titration of Aβ in cell culture supernatants. Aβ concentration, in cell culture supernatants collected at each time point, was measured by mouse anti-Aβ IgG 6E10 IgG, followed by GAM-HRP, and color development at 492 nm. *Open bar* Samples from cells incubated with 5 μM $A\beta_{1–42}$. *Full bar* Samples from cells incubated with 5 μM $A\beta_{1–42}$ and 1.6 μM Hpt. Samples were processed in triplicate. Data were expressed as mean±SEM. Significance of differences is shown. ***$p<0.001$. **c** Representative Western blot of cell lysates. Samples were fractionated by 4–20 % SDS-PAGE, under denaturing but non reducing conditions followed by Western blotting. Immunocomplexes were detected by mouse anti-Aβ IgG 6E10 IgG and GAM-HRP. **d** Representative ELISA titration of intracellular Aβ. Aβ concentration, in cell lysates collected at each time point, was measured by mouse anti-Aβ IgG 6E10 IgG, followed by GAM-HRP, and color development at 492 nm. *Open bar* Samples from cells incubated with 5 μM $A\beta_{1–42}$. *Full bar* Samples from cells incubated with 5 μM $A\beta_{1–42}$ and 1.6 μM Hpt. Samples were processed in triplicate. Data were reported as microgram of peptide per milligram of protein, and expressed as mean±SEM. Significance of differences is shown. *$p<0.05$; ***$p<0.001$. For each panel, one representative of at least three different experiments is represented

In order to further investigate the dose effect of Hpt on $A\beta_{1–42}$ clearance, U-87 MG were incubated with 5 μM $A\beta_{1–42}$ and different amounts of Hpt (0–3 μM) for 3 h. As detected by immunoblotting, the amount of peptide in cell culture supernatants increased with the increase in Hpt concentration (Fig. 4a, on the left). In agreement with this result, the level of $A\beta_{1–42}$ internalized decreased as Hpt concentration in the incubation mixture raised (Fig. 4b, on the left). In detail, the peptide level in the supernatants was found, by ELISA, significantly higher ($P<0.001$; Fig. 4a, on the right) in the presence of Hpt, at any assayed concentration (1.2-, 1.9-, and 2.2-fold in the presence of 0.5, 1.6, and 3 μM protein, respectively). The amount of internalized peptide was lower ($P<0.01$ Fig. 4b, on the right) in the presence of Hpt, at any assayed concentration (1.3-, 1.6-, and 3.5-fold in the presence of 0.5, 1.6, and 3 μM protein, respectively).

Hpt effect on $A\beta_{1–42}$ clearance was then compared with that of another extracellular chaperone, ApoE, which was previously reported to influence Aβ clearance by astrocytes (Verghese et al. 2013). U-87 MG were incubated (1.5–20 h) with 5 μM $A\beta_{1–42}$, in the absence or presence of 1.6 μM Hpt, or 1.6 μM LipoE3, or 1.6 μM Hpt and 1.6 μM LipoE3. Aliquots of supernatants and cell lysates were then analyzed both by immunoblotting and by ELISA for measuring the amount of $A\beta_{1–42}$. Western blotting analysis demonstrated that, at each time point, the amount of $A\beta_{1–42}$ was lower in the cell culture supernatant collected from cells incubated with peptide alone (Figure S2, panel A) than in the supernatants obtained from cells incubated in the presence of Hpt, or LipoE3, or Hpt and LipoE3. In particular, the peptide concentration, assessed by ELISA, was higher in the supernatant collected after 1.5, 3, 6, or 20 h in the presence of Hpt (1.54 ±0.01-fold, $P<0.01$), or LipoE3 (1.86±0.05-fold, $P<0.01$), or Hpt and LipoE3 (2.25±0.09-fold, $p<0.001$) than in that from cells incubated with $A\beta_{1–42}$ alone (Figure S2, panel b). Further, the peptide concentration was higher in the supernatant collected after incubation in the presence of both proteins than in that from cells incubated with Hpt alone ($p<0.001$), or

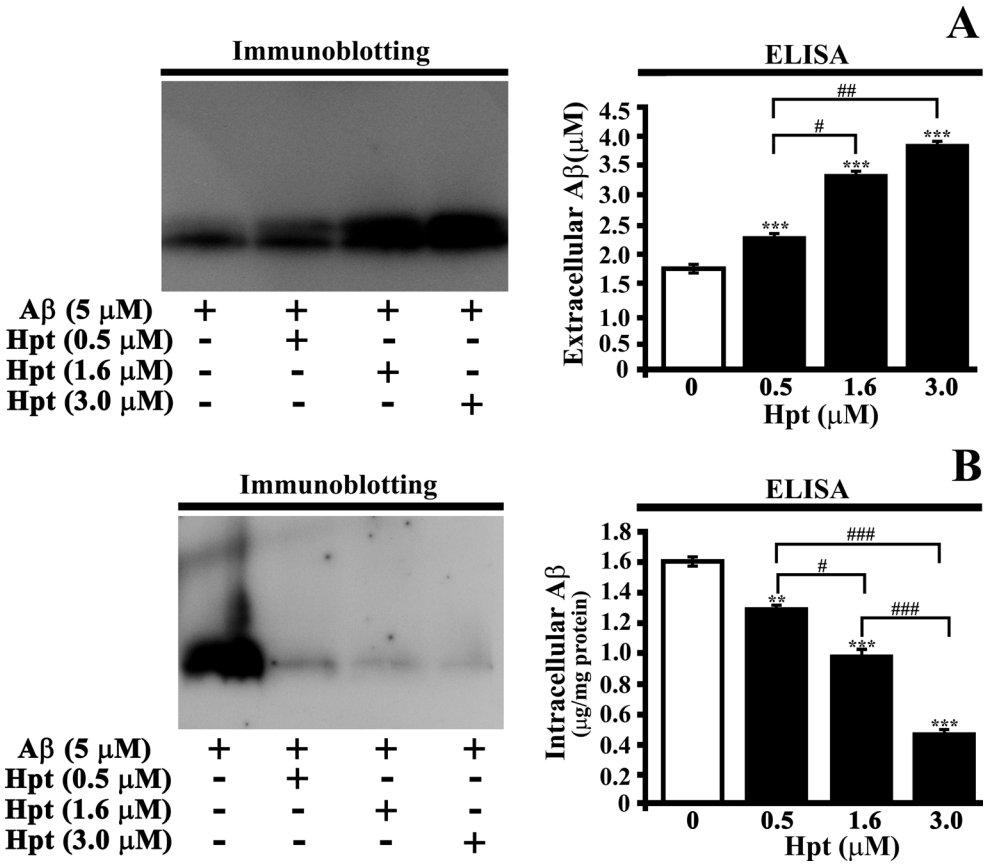

Fig. 4 Effect of Hpt concentration on astrocyte-mediated $A\beta_{1–42}$ clearance. U-87 MG (96-well plates; 15,000 cells/well) were incubated (3 h, 37 °C) in DMEM containing 5 μM $A\beta_{1–42}$ and different amounts of Hpt (0, 0.5, 1.6, or 3 μM). Media samples and cell lysates were analyzed by Western blotting (*left*) and by ELISA (*right*) for measuring Aβ concentration. **a** *left* Representative Western blot of cell culture supernatants. Samples were fractionated by 4–20 % SDS-PAGE, under denaturing but non-reducing conditions followed by Western blotting. Immunocomplexes were detected by mouse anti-Aβ IgG 6E10 IgG and GAM-HRP. *right* Representative ELISA titration of extracellular Aβ. Aβ concentration in cell culture supernatants was measured by mouse anti-Aβ IgG 6E10 IgG, followed by GAM-HRP, and color development at 492 nm. *Open bar* Samples from cells incubated with 5 μM $A\beta_{1–42}$. *Full bar* Samples from cells incubated with 5 μM $A\beta_{1–42}$ and Hpt. Samples were processed in triplicate. Data were expressed as mean±SEM. Significance of differences is shown. $^{***}p<0.001$ vs 0 μM Hpt. $^{\#}p<0.05$, $^{\#\#}p<0.01$. **b** *left* Representative Western blot of cell lysates. Samples were fractionated by 4–20 % SDS-PAGE, under denaturing but non reducing conditions followed by Western blotting. Immunocomplexes were detected by mouse anti-Aβ IgG 6E10 IgG and GAM-HRP. *right* Representative ELISA titration of intracellular Aβ. Aβ concentration in cell lysates was measured by mouse anti-Aβ IgG 6E10 IgG, followed by GAM-HRP, and color development at 492 nm. *Open bar* Samples from cells incubated with 5 μM $A\beta_{1–42}$. *Full bar* Samples from cells incubated with 5 μM $A\beta_{1–42}$ and Hpt. Samples were processed in triplicate. Data were reported as microgram of peptide per milligram of protein and expressed as mean±SEM. Significant differences are shown. $^{**}p<0.01$ vs 0 μM Hpt; $^{***}p<0.001$ vs 0 μM Hpt. $^{\#}p<0.05$; $^{\#\#\#}p<0.001$. For each panel, one representative of at least three different experiments is represented

with LipoE alone ($p<0.05$). Our results suggest that both proteins act reducing the removal of $A\beta_{1–42}$ from the culture medium and demonstrate that LipoE effect was significantly higher ($p<0.05$) than Hpt effect within 3 h of incubation. Accordingly, the amount of peptide internalized was higher ($p<0.001$) when cells were incubated with peptide alone (Figure S2, panel c) than after incubation with Hpt, or LipoE3, or Hpt and LipoE3, at each time point.

Aβ Effect on Cell Survival in the Presence of Hpt Aβ is toxic towards various cultured cerebral cells including neurons (Pike et al. 1993; White et al. 1998; Manelli et al. 2007) and astrocytes (Brera et al. 2000). Aβ-induced cell dysfunction and death was suggested to strongly depend on the peptide ability to interact with plasma membrane (Williams and Serpell 2011). Since we found that Hpt interferes with Aβ uptake by astrocytes, we investigated whether Hpt might hence modulate its cytotoxic effect.

$A\beta_{1–42}$ concentrations affecting U-87 MG survival were determined in a preliminary experiment, by incubating cells (24 h) with different amounts of peptide (0–15 μM). As shown in Fig. 5a, $A\beta_{1–42}$ treatment decreased the cell survival in a dose-dependent manner. In particular, the treatment with 3 or 6 μM $A\beta_{1–42}$ reduced cell viability to 77 % ($p=0.04$) or 55 % ($p=0.003$), respectively. Data were analyzed by non-linear regression, and the EC50 value was 13.8 μM.

The $A\beta_{1–42}$ concentration 6 μM was chosen for further experiments, carried out by incubating U-87 MG with $A\beta_{1–42}$

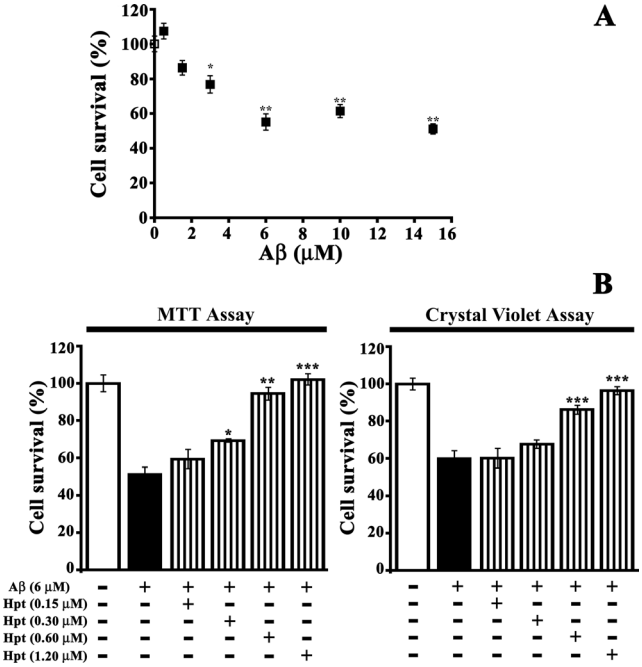

Fig. 5 Aβ effect on cell survival in the presence of Hpt. A. U-87 MG were incubated into 96-well plate (20 h; 5000 cells per well) in DMEM containing different amounts of $A\beta_{1–42}$ (0, 0.5, 1.5, 3, 6, 10, or 15 μM). Cell survival was evaluated by MTT assay, and it was expressed as percentage of viability of the control (cells cultured in the absence of Aβ; *open square*). Significant differences from control are indicated (*$p<0.05$; **$p<0.01$). Data are reported as mean±SEM. **b** U-87 MG were incubated into 96-well plate (20 h; 5000 cells per well) in DMEM containing 6 μM $A\beta_{1–42}$ and different amounts of Hpt (0, 0.15; 0.3, 0.6, or 1.2 μM; *bars with vertical line*). Cell survival was evaluated by MTT assay (on the *left*) and by Crystal Violet assay (on the *right*) and expressed as percentage of viability of the control (cells cultured without Aβ and Hpt; *open bar*). Significant differences from cells treated by $A\beta_{1–42}$ (*full bar*) are indicated (*$p<0.05$; **$p<0.001$; ***$p<0.0001$). Data are reported as mean±SEM

in the presence of different amounts of Hpt (0–1.2 μM). MTT analysis demonstrated that the treatment with 6 μM Aβ reduced cell metabolic activity to 51.3±3.8 % of the control ($p=0.001$), and this activity was significantly higher ($p\leq0.05$; Fig. 5b) when incubation with the peptide was performed in the presence of Hpt. In particular, it was restored to 69.3±1 % of the control ($p<0.05$) by 0.3 μM Hpt, to 94.4±3.4 % of the control ($p<0.001$) by 0.6 μM Hpt, and it was completely rescued when treatment was carried out in the presence of 1.2 μM Hpt. In agreement with this result, Crystal Violet assay demonstrated that cell survival was restored to 87±3.1 % and to 97.8±2.8 % of the control ($p=0.001$) by 0.6 and 1.2 μM Hpt, respectively. These data suggest that Hpt, by binding Aβ, might play a protective effect, by limiting the peptide-dependent impairment of cell metabolic activity and survival.

Hpt Synthesis and Secretion by U-87 MG

Aβ peptides were reported to activate astrocytes and promote secretion of inflammatory mediators, which in turn contribute to the pathogenesis of neurodegenerative disorders, such as AD (Wyss-Coray et al. 2003; Carrero et al. 2012; Medeiros and Laferla 2013). Hence, we investigated whether Aβ may influence Hpt production by astrocytes as well. U-87 MG cells were incubated (10 h) in DMEM containing different amounts of $A\beta_{1–42}$ (0, 3, or 6 μM), and aliquots of supernatants and cell lysates were then analyzed by electrophoresis and Western blotting. Hpt was detected in both supernatants and cell lysates collected from cells not exposed to $A\beta_{1–42}$, thus suggesting that these cells synthesize Hpt (Fig. 6a and b, lane 1). The treatment with 3 μM $A\beta_{1–42}$ did not affect Hpt production. Conversely, the supernatants collected from cells treated with 6 μM $A\beta_{1–42}$ showed a higher amount of Hpt (about 1.6-fold, $p=0.01$; Fig. 6a, lane 3) compared to supernatants from control cells. Accordingly, Hpt level in lysates from cells treated with 6 μM $A\beta_{1–42}$ was higher (1.8-fold, $P<0.01$; Fig. 6b, lane 3) than in those from untreated cells. These results suggest that $A\beta_{1–42}$ promotes Hpt synthesis and secretion by astrocytes.

Discussion

Due to their proximity with senile plaques and to their capacity to internalize and degrade Aβ deposit, astrocytes were suggested to play an important role in AD pathology, although the endocytic pathways in these cells are poorly understood (Funato et al. 1998; Thal et al. 2000; Lasagna-Reeves and Kayed 2011; Thal 2012). In order to find new therapeutic strategies, it is important to understand factors that govern Aβ metabolism and how they lead to Aβ accumulation. The amount of Aβ accumulated in the extracellular space depends on the balance between its synthesis and its clearance (Huang and Mucke 2012). Many proteins have been shown to bind Aβ and influence its aggregation and clearance. The discovery of abundant extracellular chaperones that are capable of influencing early events in the amyloid cascade has generated considerable interest in their role in disease pathogenesis and, potentially, as targets for intervention. Besides ApoJ and ApoE, other plasma and CSF proteins, such as α2 macroglobulin, Hpt, serum amyloid-P, and transthyretin, have been identified as extracellular chaperones, able of influencing Aβ aggregation and/or toxicity (Veerhuis et al. 2003; Yerbury et al. 2005; Wilson et al. 2008). It is remarkable how often these chaperones have been identified as altered in AD. Very recently, various research groups reported, by different experimental approaches, that some AAPs, particularly ApoJ and ApoE, can negatively affect, in specific conditions, Aβ clearance by glial cells (Kim et al. 2011; Bien-Ly et al. 2012; Verghese et al. 2013; Mulder et al. 2014).

We here provide evidence, for the first time, that Hpt significantly inhibits the uptake of Aβ by astrocyte cell line.

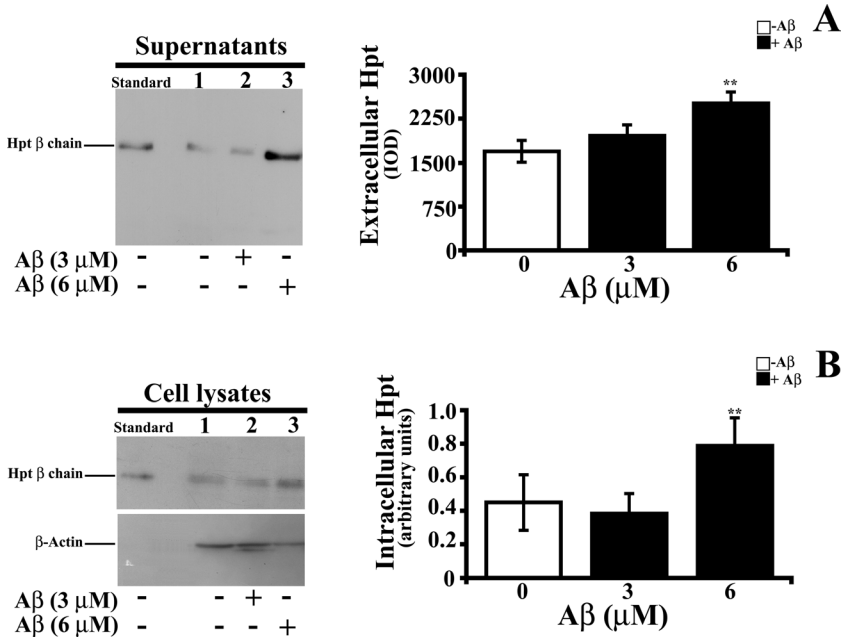

Fig. 6 Hpt synthesis and secretion by U-87 MG. U-87 MG were incubated into 24-well plate (10 h; 100,000 cells/well) in DMEM containing different amounts of $A\beta_{1-42}$ (0, 3, or 6 μM). At the end of incubation, media samples (**a**) and cell lysates (**b**) were analyzed by electrophoresis on 15 % polyacrylamide gel, under denaturing and reducing conditions, and Western Blotting. **a** Representative Western blot of cell culture supernatants (on the *left*). Immunocomplexes were detected by rabbit anti-human Hpt IgG and GAR-HRP. Quantitative densitometry of Hpt was carried out and band intensities were calculated (shown on the *right*). The data represent the mean±SEM. Significant difference from control (cells cultured in the absence of Aβ; *open bar*) is shown. $^{**}p<0.01$. **b** Representative Western blot of cell lysates (on the *left*). Immunocomplexes were detected by rabbit anti-human Hpt IgG and GAR-HRP. After Hpt detection, the membrane was stripped for reprobing with anti-β-actin. Quantitative densitometry of Hpt and β-actin was carried out, and band intensities were calculated. Hpt concentration is shown relative to β-actin level (arbitrary units, on the *right*). The *data* represent the mean±SEM. Significant difference from control (cells cultured in the absence of Aβ; *open bar*) is shown. $^{**}p<0.01$

Hpt was reported to bind Aβ (Yerbury et al. 2005; Spagnuolo et al. 2014b) and reduce peptide aggregation (Yerbury et al. 2009). We actually found that Hpt was able to impair peptide aggregation in our experimental conditions (Aβ/Hpt molar ratio 3:1; data not shown). Therefore, Hpt may influence Aβ uptake from astrocytes by preventing the binding of Aβ on cell surface and/or by reducing peptide aggregation. As matter of the fact, it was recently reported that the ability of U-87 to internalize aggregated Aβ exceeds that of monomeric Aβ (Mulder et al. 2014). Whether Hpt acts either by competing with Aβ for the same pathways or through other effects on Aβ clearance requires further studies. Importantly, we demonstrate here that Hpt might play a protective effect by inhibiting Aβ internalization. Indeed our data demonstrate that Hpt prevents the peptide-dependent impairment of cell metabolic activity, thus preserving glial cell survival. This neuroprotective action might either depend on the Hpt ability of binding Aβ, which could interfere with the peptide interaction with its receptor, and/or on the Hpt ability of reducing peptide aggregation. It cannot be excluded that Hpt impairs Aβ entry in astrocytes while promoting its clearance through BBB or other brain cells such as microglia, or endothelial cells. Beneficial effects of Hpt were already reported, since it plays a critical role in defending neurons from damage, by neutralizing iron-rich hemoglobin released into the brain parenchyma, after intracerebral hemorrhage (Zhao et al. 2009). In addition, we previously demonstrated that Hpt is able to protect apolipoprotein A-I and ApoE from oxidative damage (Salvatore et al. 2007, 2009). Although the exact role of Hpt in AD pathology remains to be elucidated, our data suggest that a local release of Hpt might contribute to modulate Aβ uptake and clearance by astrocytes.

Age-related variations of Hpt might affect Aβ metabolism and cell survival in brain. Indeed, we recently reported that Hpt level significantly increases during aging in rat hippocampus, which is the region massively damage in AD (Spagnuolo et al. 2014a). The increase in Hpt with age, in this brain compartment, might affect the astrocyte ability to incorporate Aβ, thus influencing the extracellular concentration of this peptide. This might promote Aβ aggregation pathways in the long term but might also increase the stability of Aβ in the extracellular compartment in situations in which the beneficial physiological roles of this peptide are necessary for brain health. Indeed, the dual role of Aβ is long far known, since it can play a neuroprotective role, by promoting neuronal growth and survival (Bishop and Robinson 2004; Giuffrida et al. 2010), and also protecting against excitotoxic death (Luo et al. 1996; Giuffrida et al. 2009), but, at high concentrations,

it can cause neuronal degeneration in mature neurons (Yankner et al. 1990). In addition, it was shown to serve a double prooxidant/antioxidant role (Kontush et al. 2001; Butterfield 2002; Nadal et al. 2008; Baruch-Suchodolsky et al. 2009) and to bind and remove harmful substances by blocking them in plaques (Bishop and Robinson 2002; Robinson and Bishop 2002). Aβ ability to enhance synaptic plasticity and memory was reported as well (Puzzo and Arancio 2013). From these studies, the fascinating hypothesis arises that, in the healthy brain, physiological Aβ concentrations are necessary for the correct functioning of the cerebral compartment.

Interestingly, U-87 MG cell line is here reported to enhance Hpt production and release when challenged with Aβ. This finding suggests that these cells might undertake specific pathways to produce extracellular chaperones, such as Hpt, which are able to hold Aβ in the extracellular compartment and, at the same time, limit its pathological aggregation. The increase in Hpt level might affect brain pathophysiology, modulating Aβ internalization and protecting cells from cholesterol toxicity or oxidative stress. On the other hand, it cannot be excluded that enhanced Hpt levels, by limiting Aβ uptake by astrocytes, might represent a further way by which inflammation worsens the onset and rate of progression of neurodegeneration. We cannot assess, to date, whether the positive effects of Hpt in brain outweigh the negative effects, or vice versa. How exactly Hpt influences Aβ metabolism, neuronal functions, and survival remains to be determined. Furthermore, further studies are required to highlight whether Hpt undergo changes during the onset and progression of AD. The study of the risk of developing AD or the research of new therapeutic strategies should be focused not only on the analysis of Aβ concentration but also on the level of critical ligands, such as Hpt, able to influence peptide aggregation or clearance.

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