ARTICLE
Journal of Cellular Biochemistry 115:2116–2122 (2014)

Journal of Cellular Biochemistry

Platelet-Activating Factor Mediates the Cytotoxicity Induced by W7FW14F Apomyoglobin Amyloid Aggregates in Neuroblastoma Cells

Ivana Sirangelo,[1]* Alfonso Giovane,[1] Rosa Maritato,[1] Nunzia D'Onofrio,[1] Clara Iannuzzi,[1] Antonio Giordano,[2,3] Gaetano Irace,[1] and Maria Luisa Balestrieri[1]

[1]*Department of Biochemistry, Biophysics and General Pathology, Second University of Naples, Naples, Italy*
[2]*Sbarro Research Institute, College of Science and Technology, Temple University, Philadelphia, Pennsylvania, USA*
[3]*Department of Medicine, Surgery & Neuroscience, University of Siena, Siena, Italy*

ABSTRACT

W7FW14F apomyoglobin (W7FW14F ApoMb) amyloid aggregates induce cytotoxicity in SH-SY5Y human neuroblastoma cells through a mechanism not fully elucidated. Amyloid neurotoxicity process involves calcium dyshomeostasis and reactive oxygen species (ROS) production. Another key mediator of the amyloid neurotoxicity is Platelet-Activating Factor (PAF), an inflammatory phospholipid implicated in neurodegenerative diseases. Here, with the aim at evaluating the possible involvement of PAF signaling in the W7FW14F ApoMb-induced cytotoxicity, we show that the presence of CV3899, a PAF receptor (PAF-R) antagonist, prevented the detrimental effect of W7FW14F ApoMb aggregates on SH-SY5Y cell viability. Noticeably, we found that the activation of PAF signaling, following treatment with W7FW14F ApoMb, involves a decreased expression of the PAF acetylhydroase II (PAF-AH II). Interestingly, the reduced PAF-AH II expression was associated with a decreased acetylhydrolase (AH) activity and to an increased sphingosine-transacetylase activity (TA_S) with production of N-acetylsphingosine (C_2-ceramide), a well known mediator of neuronal caspase-dependent apoptosis. These findings suggest that an altered PAF catabolism takes part to the molecular events leading to W7FW14F ApoMb amyloid aggregates-induced cell death. J. Cell. Biochem. 115: 2116–2122, 2014.

KEY WORDS: PAF; AMYLOID AGGREGATES; AMYLOID CYTOTOXICITY

Protein misfolding and subsequent aggregation is a process underlying several neurodegenerative diseases such as Alzheimer's, Huntington's chorea and Parkinson's disease, characterized by the presence of deposits of ordered protein aggregates, namely amyloid fibrils, in the affected tissues [Dobson, 2003; Luheshi and Dobson, 2009]. These protein aggregates do not share any sequence identity or structural homology to each other [Invernizzi et al., 2012]. An increasing number of proteins with no link to protein deposition diseases has been found to form, under some in vitro conditions, fibrillar aggregates with morphological, structural, and tinctorial properties of amyloid fibrils [Guijarro et al., 1998; Litvinovich et al., 1998; Chiti et al., 1999; Vilasi et al., 2008, 2011; Infusini et al., 2012], suggesting that the amyloid aggregation might be a generic property of polypeptide chains independent of the specific amino acid sequences. W7FW14F apomyoglobin (W7FW14F ApoMb) is a protein that, although not related to any amyloid disease, is able to form amyloid aggregates in physiological condition quite similar to those formed from proteins directly involved in amyloid pathologies [Sirangelo et al., 2002, 2004; Iannuzzi et al., 2013a,b]. Similarly to other amyloidogenic proteins, the cytotoxicity of W7FW14F ApoMb aggregates does not lie in the mature fibrils but rather in the early oligomer intermediates [Sirangelo et al., 2004; Sirangelo and Irace, 2010; Ortore et al., 2011]. Amyloid oligomers are considered to possess a structural motif and/or physicochemical properties that impart toxicity, irrespective of the amino acid sequence [Bucciantini et al., 2004]. Several data reinforce the idea that amyloid oligomers are really formed in vivo and are directly associated with cell/tissue impairment [Koffie et al., 2009].

Abbreviations: AH, acetylhydrolase; PAF, Platelet-activating factor; PAF-AH II, PAF acetylhydroase II; PAF-R, PAF receptor; ROS, reactive oxygen species; TA_L, lysophospholipid transacetylase; TA_S, sphingosine-transacetylase; W7FW14F ApoMb, W7FW14F apomyoglobin.

The authors have no conflict of interests to declare.

*Correspondence to: Dr. Ivana Sirangelo, Department of Biochemistry, Biophysics and General Pathology, Second University of Naples, via L. De Crecchio 7, 80138, Naples, Italy. Email: ivana.sirangelo@unina2.it

Manuscript Received: 18 June 2014; Manuscript Accepted: 18 July 2014

Accepted manuscript online in Wiley Online Library (wileyonlinelibrary.com): 23 July 2014

DOI 10.1002/jcb.24888 • © 2014 Wiley Periodicals, Inc.

Reactive oxygen species (ROS) production and Platelet-Activating Factor (PAF) play a central role in the events mediating the toxic action of amyloid aggregates, but the precise molecular mechanism is not still completely understood [Mattson, 1999; Butterfield et al., 2001; Kadowaki et al., 2005; Glabe and Kayed, 2006; Demuro et al., 2010; Jomova et al., 2010]. In hippocampal neuronal cultures, both H_2O_2 and PAF were able to reproduce each of the events induced by oligomeric Aβ1–42, including triggered sustained Ca^{2+} influx via N-methyl-D-aspartic acid receptors, enhanced extracellular accumulation of glutamate, and increase in cytosolic free Ca^{2+} [Shi et al., 2010]. PAF, a potent inflammatory lipid, plays crucial roles in the neuronal damage and death in various pathological conditions [Prescott et al., 2000; Tong et al., 2001; Xu and Tao, 2004; Bate et al., 2004a; Goracci et al., 2009; Balestrieri et al., 2010; Farooqui et al., 2010]. The PAF-induced neuronal toxicity is mainly characterized by increased levels of PAF due to the upregulation of its biosynthesis or downregulation of its degradation [Goracci et al., 2009]. An increased PAF production in the brain, due to the activation of the phospholipase A_2 by Aβ peptides, has been implicated in Alzheimer disease [Bate et al., 2004a; Kriem et al., 2005; Farooqui and Horrocks, 2006; Bate et al., 2008; Sanchez-Mejia et al., 2008; Sirangelo et al., 2013]. High concentrations of PAF were shown to be able to reproduce many of the effects of Aβ peptides on neurons [Bate et al., 2006, 2008]. Moreover, neurons pre-treated with PAF receptor (PAF-R) antagonists were resistant to Aβ1–42 [Bate et al., 2004a,b, 2006, 2008; Li et al., 2009]. On the other hand, increased levels of PAF due to downregulation of its degradation catalyzed by the PAF-acetylhydrolase (AH) type I (PAF–AH I) or II (PAF–AH II) are critically involved in neurodegenerative diseases [Goracci et al., 2009]. In particular, the PAF–AH II contains three separate catalytic activities, namely acetylhydrolase (AH), lysophospholipid transacetylase (TA_L), and sphingosine transacetylase (TA_S), through which PAF functions are modified by hydrolyzing PAF to lyso-PAF (AH) or converting PAF to acyl analogs of PAF (TA_L) or N-acetylsphingosine (TA_S) [Balestrieri et al., 2010].

The cytotoxicity induced by W7FW14F ApoMb amyloid oligomers is reported to be closely related to oxidative stress, as well as perturbation of calcium homeostasis, apoptotic and survival pathways activation, and membrane damage [Sirangelo et al., 2009; Vilasi et al., 2013]. In this work, we examined the possible involvement of PAF signaling pathway in the cytotoxicity induced by W7FW14F ApoMb amyloid aggregates. To this end, we evaluated the SH-SY5Y human neuroblastoma cell viability after exposure to W7FW14F ApoMb in the presence of PAF-R antagonist, CV3988, and determined PAF–AH II expression and activities.

MATERIALS AND METHODS

PROTEIN PURIFICATION AND AGGREGATE FORMATION

W7FW14F ApoMb mutant was expressed and purified essentially as described elsewhere [Sirangelo et al., 2002; Vilasi et al., 2011]. Briefly, the protein was expressed in the Escherichia coli M15 [pREP4] strain as N-terminal His-tagged form and then purified via affinity chromatography on Ni^{2+} nitrilotriacetic-acid resin (Qiagen, Milan, Italy) in denaturing conditions. Protein purity was checked by SDS–PAGE analysis. Refolding was achieved by removing the denaturant by dialysis against 10 mM NaH_2PO_4, pH 2.0, containing decreasing concentrations of urea. Protein concentration was determined under denaturing conditions, and absorption was measured at 275 nm. The molar extinction coefficient at 275 nm calculated from tyrosine content was 3750 M^{-1} cm^{-1}. Prefibrillar aggregates were obtained adjusting the pH of a 40 μM protein solution at the neutrality. The W7FW14F ApoMb aggregation kinetics at this concentration, pH and ionic strength has been extensively studied [Vilasi et al., 2008, 2010, 2011]. The time corresponding to the formation of toxic prefibrillar aggregates was verified by analyzing the fluorescence of Thioflavin T incubated with the sample at the beginning of the aggregation.

CELL CULTURE AND INCUBATION WITH W7FW14F ApoMb AMYLOID AGGREGATES

SH-SY5Y cells (human neuroblastoma cell line, American Type Culture Collection) were cultured in DMEM/HAM′S F12 (1:1) medium supplemented with heat inactivated 10% FBS, 20 mM HEPES, 100 U/ml penicillin, 100 mg/ml streptomycin, 1% L-glutamine and 1% sodium pyruvate in a 5.0% CO_2 humidified environment at 37 °C. The cells were plated at a density of 100.000 cells/well on 12-well plates in 1 ml of medium. After 24 h, W7FW14F ApoMb amyloid aggregate samples (40 μM) were mixed 1:1 v/v with cell media and incubated for a time course spanning 6 h. Cells in culture medium without protein served as control.

When PAF-R antagonist was used, cells were pre-treated with CV3988 (10 μM) for 2 h at 37 °C. After 2 h, CV3988 was removed, cells were washed twice with HBSS-10 mM Hepes, and treated with W7FW14F ApoMb amyloid aggregates for 15 min in serum-free media. Control cells were cultured for 15 min in serum-free media [Balestrieri et al., 2010].

CELL VIABILITY ASSAY

Cell viability was determined by mitochondrial dehydrogenase activity, by using XTT (sodium 3′-[1-(phenylaminocarbonyl)- 3,4-tetrazolium]-bis (4-methoxy-6-nitro) benzene sulfonic acid hydrate) assay (Roche) following manufacture′s protocol. Absorbance rate was measured at 490 nm using a micro plate reader (BIO–RAD). Cell growth data are expressed as percentage of the control.

DETECTION OF INTRACELLULAR ROS

Intracellular ROS were detected by means of an oxidation-sensitive fluorescent probe 2′,7′-dichlorofluorescin diacetate (DCFH-DA, Sigma–Aldrich Co., St. Louis, MO). SH-SY5Y cells were grown in a six-well plates, pre-incubated with DCFH-DA for 30 min and then incubated with W7FW14F ApoMb prefibrillar aggregates for 15, 30, 60, 180 and 360 min. Untreated cells were used as controls. After incubation, cells were washed twice with PBS buffer and then lysated with Tris-HCl 0.5 M, pH 7.6, 1% SDS. DCFH-DA, initially non-fluorescent, is converted by oxidation to the fluorescent molecule 2′,7′-dichlorofluorescein (DCF). DCF fluorescent intensity was then quantified on a Perkin Elmer Life Sciences LS 55 spectrofluorimeter using an excitation wavelength of 488 nm and an emission wavelength of 530 nm. The fluorescence intensity was corrected

by subtracting the fluorescence value of the control cells and expressed as F-F_0.

WESTERN BLOT ANALYSIS

SH-SY5Y cells were collected, washed twice with PBS and treated with lysis buffer (Tris-HCl 50 mM pH 7.4, NP-40 1%, NaCl 150 mM, EDTA 1 mM, PMSF, 1 mM, Leupeptin 1 μg/ml, Pepstatin 1 μg/ml, Aprotinin 1 μg/ml, NaF 1 mM, Na_3VO_4 1 mM). Approximately, 15 μg of protein extract were separated by 12.5% SDS–PAGE as described [Balestrieri et al., 2010]. The gels were transblotted onto a nitrocellulose membrane, blocked with 3% bovine albumin serum in Tris-buffered saline (pH 7.4) with 0.1% Tween 20 (TBS-T) for 2 h and incubated overnight at 4 °C with antibody against PAF-R (Cayman Chemical Company) or PAF–AH II. Antibody against PAF–AH II peptide 351–365 (UniProtKB/Swiss-Prot entry Q99487) (Arg-Ala-Met-Leu-Ala-Phe-Leu-Gln-Lys-His-Leu-Asp-Leu-Lys-Glu) was from Primm s.r.l. After incubation with secondary antibodies, the signal was detected with the aid of a chemiluminescence kit. Membranes were normalized with a polyclonal antibody against tubulin protein (GTU-88) (Sigma–Aldrich). Semiquantitative densitometry of Western blots was performed by using a Scan LKB (Amersham Pharmacia) [Balestrieri et al., 2010].

CONFOCAL LASER-SCANNING MICROSCOPY

SH-SY5Y cells were fixed with 3% paraformaldehyde for 20 min and were permeabilized with 0.1% Triton X-100 in PBS for 10 min at room temperature. Cells were incubated overnight at 37 °C with specific antibodies against PAF-R (Cayman Chemical Company) (1:500) and vimentin (1:1000; Sigma–Aldrich) followed by incubation with secondary antibodies conjugated to Alexafluor 633 (1:1000) or Alexafluor 488 (1:1000) for 1 h at room temperature. Microscopy analysis were performed using Zeiss LSM 510 confocal microscope equipped with a plan-apochromat 63X (NA 1.4) oil immersion objective, as previously described [Balestrieri et al., 2010].

IN VITRO ENZYME ASSAYS

Cell homogenates were prepared by sonicating SH-SY5Y cells at 30% power out-put for 15 s × 5 times in the homogenizing buffer (0.25 M sucrose, 100 mM Tris-HCl pH 7.3, 1 μg/ml leupeptin, and 1 mM dithiotreitol). The lysophospholipid transacetylase (TA_L), the sphingosine transacetylase (TA_s), and PAF-acetylhydrolase (AH) activities were determined as previously described [Balestrieri et al., 2003]. Briefly, TA_L and TA_S activities were determined using [^{3}H] acetyl-PAF as acetyl donor and lyso-glycerophosphoethanolamine (LPE) or sphingosine as substrate acceptors, respectively. The extracted lipids were separated by thin layer chromatography and the radioactivity of the areas corresponding to [^{3}H]acetyl-PAF, [^{3}H] acetyl-phosphoethanolamine, and [^{3}H]acetyl-sphingosine were determined by liquid scintillation counting [Balestrieri et al., 1997, 2003; Lee et al., 2001].

STATISTICAL ANALYSIS

Data are expressed as mean ± SD. Differences were assessed by two-tailed Student's *t*-test with unequal variance. *P*-value less than 0.05 was considered to be significant.

RESULTS

THE EFFECT OF CV3988 ON W7FW14F ApoMb-ALTERED CELL VIABILITY

We first evaluated the oxidative stress induced by W7FW14F ApoMb amyloid oligomers, known to occur at early time of cell exposure to aggregates [Sirangelo et al., 2009; Vilasi et al., 2013]. Results indicated that exposure of SH-SY5Y cells to W7FW14F ApoMb

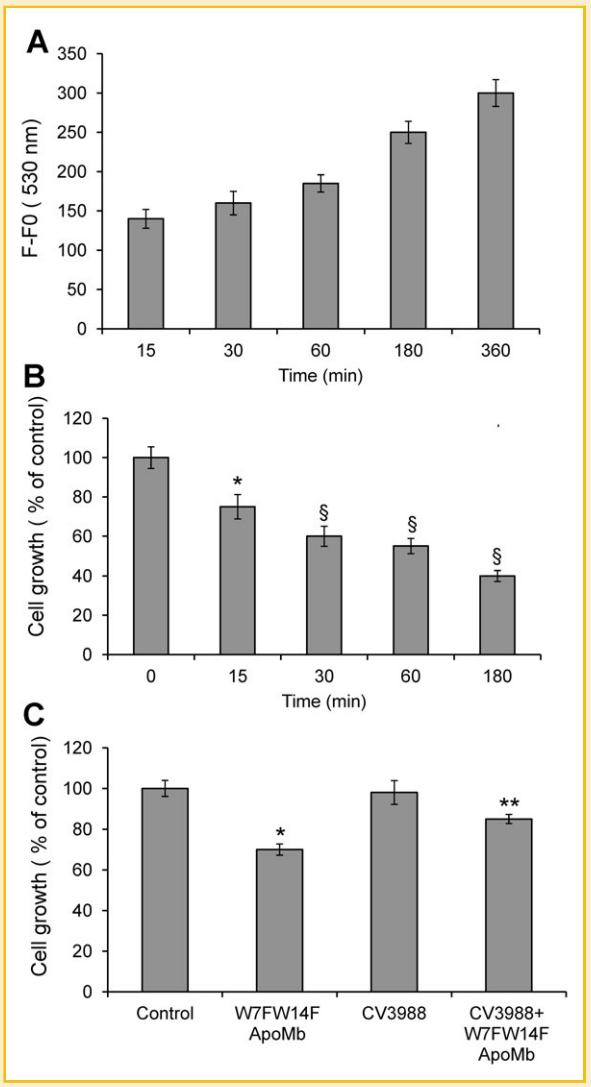

Fig. 1. ROS detection and cell growth evaluation. (A), Levels of ROS in SH-SY5Y cells treated with W7FW14F ApoMb aggregates for indicated times. ROS levels were determined by the DCFH-DA assay as described in Materials and Methods. The fluorescence intensity was corrected by subtracting the fluorescence value of the control cells and expressed as F-F_0. (B), Cell viability of SH-SY5Y cells treated with W7FW14F ApoMb aggregates up to 180 min was determined by XTT assay. (C), Cell viability of SH-SY5Y cells after 15 minutes of exposure to W7FW14F ApoMb aggregate in the absence and in the presence of PAF-R antagonist, CV3988. Results are mean ± SD of five separate experiments carried out in triplicate with *$P < 0.05$ and §$P < 0.01$ versus time zero (Panel B), *$P < 0.05$ versus control and **$P < 0.05$ versus W7FW14F ApoMb-treated cells (Panel C).

aggregates up to 360 min led to a significant increase of intracellular ROS compared with untreated cells ($P < 0.05$ at 15, 30, and 60 min, $P < 0.01$ at 180 and 360 min) (Fig. 1, panel A). Concomitantly, a decrease of cell viability was observed up to 180 min (Fig. 1, panel B); thus confirming that the cytotoxicity of W7FW14F ApoMb prefibrillar aggregates is linked to ROS-mediated pathway [Sirangelo et al., 2009]. Cell viability after 360 min of exposure to W7FW14F was comparable to values observed at 180 min. Since it has been previously demonstrated that an early onset of apoptotic cell death occurred already after 30 min exposure of SH-SY5Y cells with W7FW14F ApoMb amyloid oligomeric aggregates [Sirangelo et al., 2009], we choosed an incubation time of 15 min to test the effect of PAF-R antagonist, CV3988, on cell viability. The results indicated that the presence of CV3899 prevented the W7FW14F ApoMb aggregate-induced decrease of cell viability (Fig. 1, panel C) and suggesting that the cytotoxicity might be mediated by binding of PAF to its receptor. Specifically, SH-SY5Y cell growth values, expressed as percentage of control, were 70.02 ± 2.81 ($P < 0.05$ vs. control cells) in W7FW14F ApoMb aggregate-treated cells, and 85.03 ± 2.2 in CV3988 plus W7FW14F ApoMb pre-treated cells ($P < 0.05$ vs. W7FW14F ApoMb) (Fig. 1, panel C).

DETECTION OF PAF-R ON SH-SY5Y CELLS

In order to confirm the role of the PAF-mediated signaling pathway in SH-SY5Y human neuroblastoma cell line exposed to W7FW14F ApoMb aggregates, we investigated the expression of PAF-R by confocal laser-scanning microscopy analysis and Western blot analysis. Results showed that SH-SY5Y cells express PAF-R (Fig. 2, panel A, B). Analysis of the arbitrary fluorescence units (AFU) of PAF-R showed an increased expression after 30 min of treatment with W7FW14F ApoMb aggregates (33.1 ± 2.01 AFU vs. 25.0 ± 1.9 AFU at time zero, $P < 0.05$) (Fig. 2, panel B). These results were confirmed by Western blot analysis (Fig. 2, panel C, D) which showed that the arbitrary units (AU) of PAF-R expression in SH-SY5Y after 30 min of treatment were 0.99 ± 0.03 AU compared to 0.79 ± 0.04 AU at time zero ($P<0.05$).

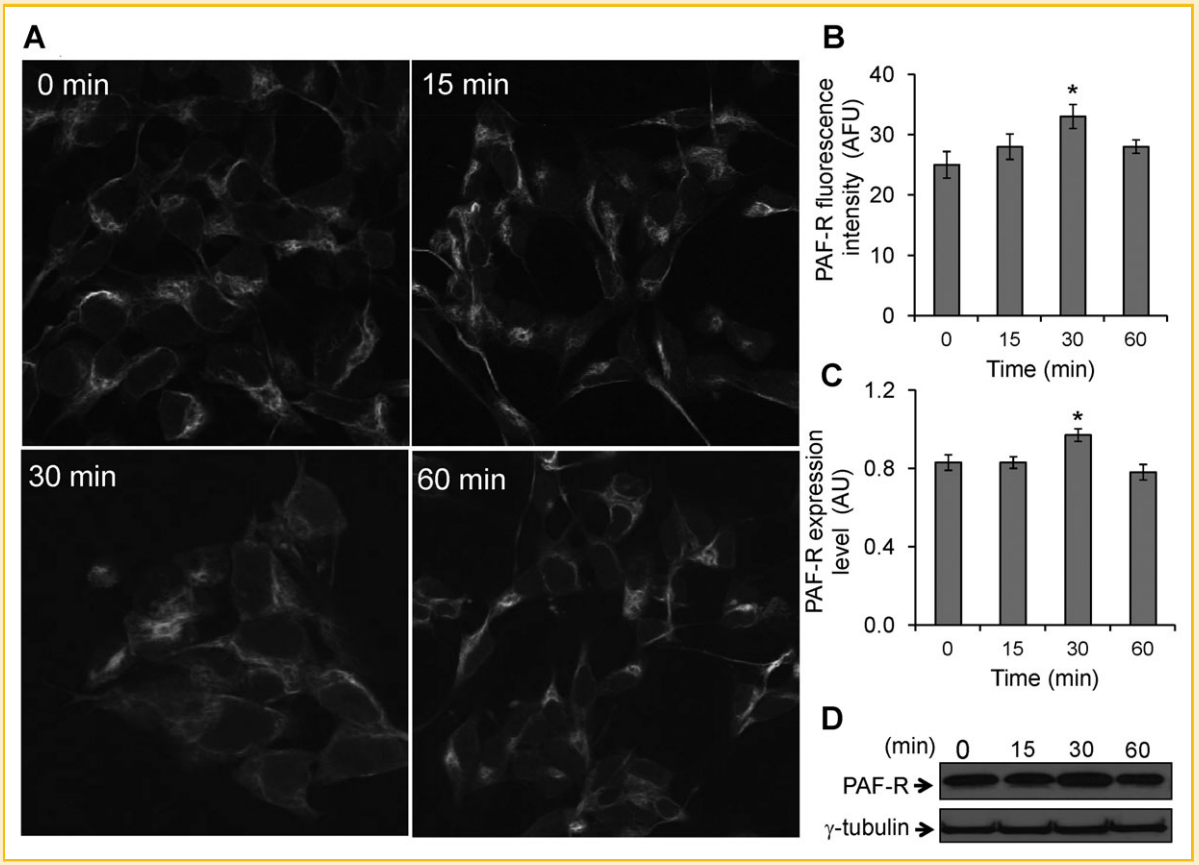

Fig. 2. PAF-R expression in SH-SY5Y cells. Cells were fixed with 3% paraformaldehyde and permeabilized with 0.1% Triton X-100 before incubation with specific antibodies against vimentin (green) and PAF-R (red). Secondary antibodies were Alexa Fluor 488 and Alexa Fluor 633. (A), Representative confocal images performed using a plan-apochromat 63X (NA 1.4) oil immersion objective. Alexa 488 and Alexa 633 fluorescence emissions were acquired in multi-track mode using BP 505–530 and LP650 filters respectively. To minimize noise each image was averaged 16 times. The fluorescence intensity was calculated with ImageJ software analysis and expressed as arbitrary fluorescence units (AFU). The mean ($n = 5$) of the unspecific fluorescence of the secondary antibodies, Alexa 488 and Alexa 633, was calculated by ImageJ software analysis and subtracted. (B), Bar graph showing the AFU for PAF-R expression. (C), Cell lysates for PAF-R expression (arbitrary units, AU) analysis by Western blot were prepared from SH-SY5Y cells treated with W7FW14F ApoMb aggregates for 0 (lane 1), 15 (lane 2), 30 (lane 3), and 60 (lane 4) min. The γ-tubulin served as loading controls. (D), Representative image of Western blot analysis. Data are mean ± SD of four experiments carried out in duplicate with $^*P < 0.05$ versus time zero.

PAF CATABOLISM IN W7FW14F ApoMb-TREATED SH-SY5Y CELLS

Increased levels of PAF, due to upregulation of biosynthetic pathways or to downregulation of its degradation through the PAF–AH, are reported in neuroinflammation, brain ischemia, and neurodegenerative diseases [Goracci et al., 2009]. Therefore, we next evaluated the time-dependent effect of W7FW14F ApoMb on the PAF–AH II expression levels (from time 0 up to 60 min). The results showed a reduced expression level at 15 min (0.9 ± 0.03 AU) compared to time zero (1.48 ± 0.06 AU) ($P < 0.05$) (Fig. 3, panel A). Interestingly, the in vitro enzymatic assays showed that stimulation with W7FW14F ApoMb induced a decrease of the AH activity at 15 min (Fig. 3, panel B) ($P < 0.05$ vs. time zero) and a concomitant increase of the TA_S activity at 15 and 30 min ($P < 0.05$ vs. time zero) (Fig. 3, panel C). The TA_L activity was not altered (Fig. 3, panel D). These results suggest that W7FW14F ApoMb induced-decrease of PAF–AH II expression is paralleled by a loss of the AH activity and an increased TA_S activity which is responsible of the production of N-acetylsphingosine (C_2-ceramide).

DISCUSSION

This study provides new knowledge into the relationship between the oxidative stress-related cytotoxicity of W7FW14F ApoMb and the PAF signaling in neuroblastoma cells. Results clearly suggest that the activation of PAF signaling takes part to the cytotoxic pathway activated by W7FW14F ApoMb in SH-SY5Y cells.

Soluble amyloid oligomers represent the primary pathologic species in degenerative diseases. These amyloid oligomers permeate membranes and share a common primary mechanism of pathogenesis. More in details, amyloid oligomers initiate a common group of downstream pathologic processes, including intracellular calcium dyshomeostasis, production of ROS, and mitochondrial dysfunction that represent key effectors of cellular dysfunction and cell death in amyloid-associated degenerative disease [Mattson, 1999; Butterfield et al., 2001; Kadowaki et al., 2005; Glabe and Kayed, 2006; Demuro et al., 2010; Jomova et al., 2010].

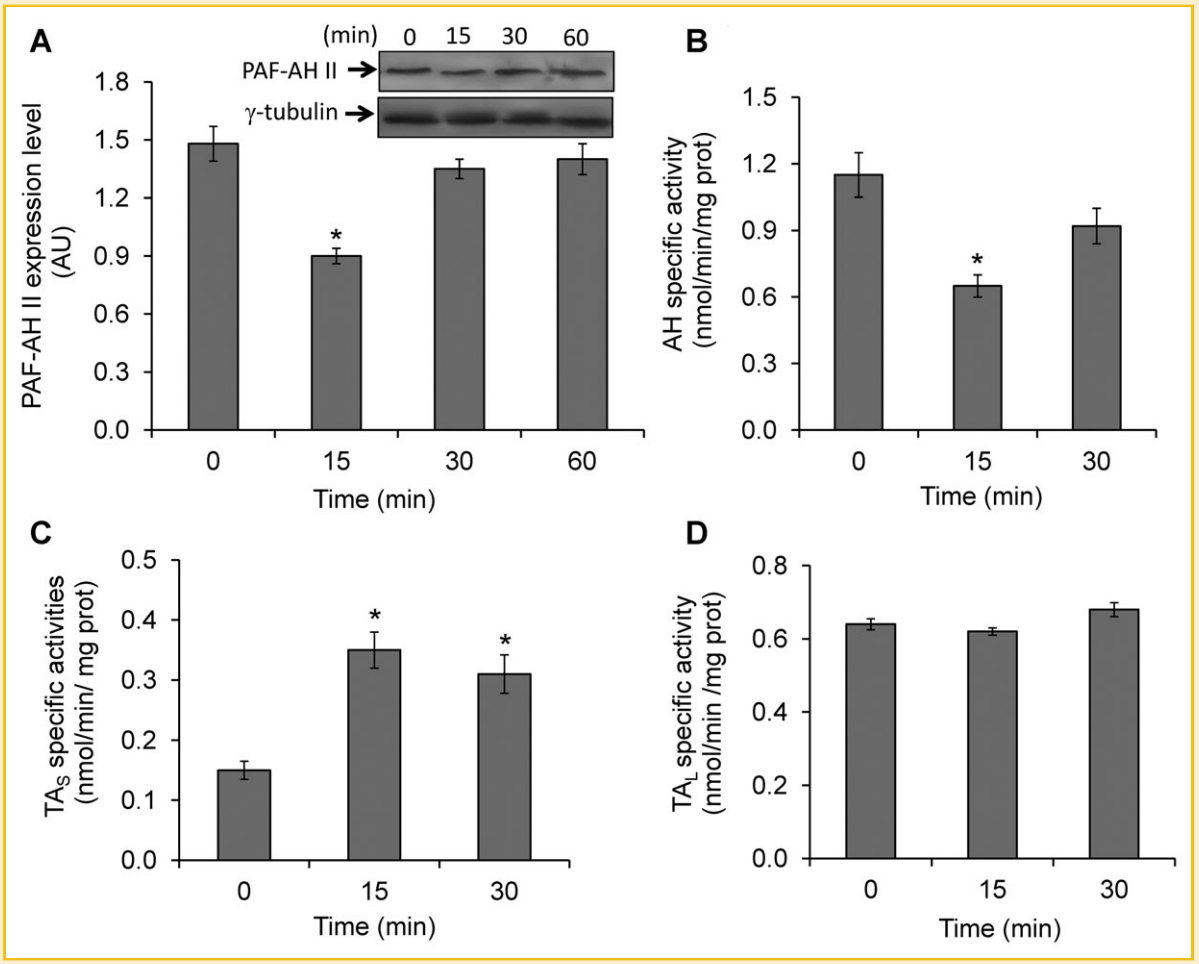

Fig. 3. PAF-AH II expression levels and enzyme assays. SH-SY5Y cells were treated with W7FW14F ApoMb aggregates for 0, 15, 30, and 60 min. At the end of incubations, Western blot analysis of PAF–AH II expression and AH, TA_S, and TA_L activities were determined on the total cell homogenates as described under Methods section. (A), Western blot analysis of PAF–AH II expression (arbitraty units, AU) from SH-SY5Y cell total homogenates after treatment with W7FW14F ApoMb aggregates for 0 (lane 1), 15 (lane 2), 30 (lane 3), and 60 (lane 4) min. The γ-tubulin served as loading controls. (B), AH, (C), TA_S, and (D), TA_L specific activities determined on the total cell homogenates were expressed as nmol/min/mg protein. Results are mean ± SD of five separate experiments in duplicate with * $P < 0.01$ versus time zero.

We demonstrated that the cytotoxicity induced by W7FW14F ApoMb is closely related to oxidative stress [Sirangelo et al., 2009; Vilasi et al., 2013]. Similarly to previous studies aimed at evaluating the mechanisms of amyloid-β-induced neurotoxicity [Bate et al., 2006; Li et al., 2009], our data show that the time-dependent decrease of cell viability elicited by W7FW14F ApoMb insult is prevented by PAF-R antagonist, CV3988, thus suggesting the involvement of PAF signaling in the apoptotic pathway activated by W7FW14F ApoMb.

The previous demonstration that PAF takes part to the mechanisms involved in Aβ-amyloid toxicity led to unravel the mechanisms involved in the regulation of the levels of this inflammatory lipid mediator in the nervous system [Goracci et al., 2009; Shi et al., 2010; Sirangelo et al., 2013]. Indeed, the activation of the enzymes of the PAF biosynthetic route, responsible for the increased levels of PAF under pathological conditions, represents a possible pharmacologic target [Bate et al., 2006]. However, the main difficulties in achieving this challenge is due to the complexity of the pathways regulating not only the synthesis, but also the degradation of PAF in central nervous system [Goracci et al., 2009]. As a consequence, relatively high concentration of PAF causes neuronal death by apoptosis [Goracci et al., 2009]. To date, specific alterations of the PAF catabolism induced by W7FW14F ApoMb are not fully unveiled.

The demonstration that SH-SY5Y cells exposed to W7FW14F ApoMb showed reduced expression and activity of PAF–AH II led us to hypothesize a possible role of this enzyme in the antioxidant defence mechanism. Indeed, PAF–AH II is known to be an antioxidant phospholipase in a number of systems since it recognizes the redox state of the cell environment, changes its localization in response to cellular requirements, and prevents damages from lipid peroxidation [Goracci et al., 2009]. More in detail, PAF–AH II translocates to the membrane during oxidative stress and inhibits oxidative stress-induced apoptosis, presumably by scavenging oxidized phospholipids [Balestrieri et al., 2001; Goracci et al., 2009]. Intriguingly, our results show that, following stimulation with W7FW14F ApoMb, the AH activity of the PAF- AH II is inhibited, whereas, the TA_s activity, which produces N-acetylsphingosine (C_2-ceramide), is significantly activated. The increased production of C_2-ceramide, a mediator of neuronal caspase-dependent apoptosis, [Hannun, 1996; Sabirzhanov et al., 2012], suggests the involvement of this pathway in the mechanism of W7FW14F ApoMb-induced apoptotic death [Sirangelo et al., 2009]. Therefore, it is likely that SH-SY5Y cells response to W7FW14F ApoMb involves an oxidative and proinflammatory mechanism with an increased production of C_2-ceramide.

In conclusion, the evidence here provided show that PAF signaling is involved in the cytotoxicity induced by W7FW14F ApoMb. Although this protein is not related to any amyloid disease, it is able to form amyloid aggregates with the same properties of proteins directly involved in amyloidosis, suggesting that a more generic mechanism may occur in the pathogenesis of amyloid diseases.

REFERENCES

Balestrieri ML, Castaldo D, Balestrieri C, Quagliuolo L, Giovane A, Servillo L. 2003. Modulation by flavonoids of PAF and related phospholipids in endothelial cells during oxidative stress. J Lipid Res 44:380–387.

Balestrieri ML, Giovane A, Milone L, Servillo L. 2010. Endothelial progenitor cells express PAF receptor and respond to PAF via Ca(2 +)-dependent signaling. Biochim Biophys Acta 1801:1123–1132.

Balestrieri ML, Servillo L, Lee T. 1997. The role of platelet-activating factor-dependent transacetylase in the biosynthesis of 1-acyl-2-acetyl-sn-glycero-3-phosphocholine by stimulated endothelial cells. J Biol Chem 272:17431–17437.

Bate C, Kempster S, Williams A. 2006. Platelet-activating factor antagonists protect amyloid beta damaged neurons from microglia-mediated death. Neuropharmacology 51:173–181.

Bate C, Salmona M, Williams A. 2004a. The role of platelet activating factor in prion and amyloid-beta neurotoxicity. Neuroreport 15:509–513.

Bate C, Salmona M, Williams A. 2004b. Ginkgolide B inhibits the neurotoxicity of prions or amyloid-beta1–42. J Neuroinflam 1:4.

Bate C, Tayebi M, Williams A. 2008. Ginkgolides protect against amyloid-beta 1–42-mediated synapse damage in vitro. Mol Neurodegener 3:1.

Bucciantini M, Calloni G, Chiti F, Formigli L, Nosi D, Dobson CM, Stefani M. 2004. Prefibrillar amyloid protein aggregates share common features of cytotoxicity. J Biol Chem 279:31374–31382.

Butterfield AD, Drake J, Pocernich C, Castegna A. 2001. Evidence of oxidative damage in Alzheimer's disease brain: Central role for amyloid β-peptide. Trends Mol Med 7:548–554.

Chiti F, Webster P, Taddei N, Clark A, Stefani M, Ramponi G, Dobson CM. 1999. Designing conditions for in vitro formation of amyloid protofilaments and fibrils. Proc Natl Acad Sci U S A 96:3590–3594.

Demuro A, Parker I, Stutzmann GE. 2010. Calcium signaling and amyloid toxicity in Alzheimer disease. J Biol Chem 285:12463–12468.

Dobson CM. 2003. Protein folding and misfolding. Nature 426:884–890.

Farooqui AA, Horrocks LA. 2006. Phospholipase A2-generated lipid mediators in the brain: The good, the bad, and the ugly. Neuroscientist 12:245–260.

Farooqui AA, Ong WY, Farooqui T. 2010. Lipid mediators in the nucleus: Their potential contribution to Alzheimer's disease. Biochim Biophys Acta 1801:906–916.

Glabe CG, Kayed R. 2006. Common structure and toxic function of amyloid oligomers implies a common mechanism of pathogenesis. Neurology 66:74–78.

Goracci G, Balestrieri ML, Nardicchi V. 2009. Metabolism and functions of platelet activating factor (PAF) in the nervous tissue. Handbook of neurochemistry and molecular neurobiology. 3rd edition. Vol 8. Springer US: Springer Science: 311–352.

Guijarro JI, Sunde M, Jones JA, Campbell ID, Dobson CM. 1998. Amyloid fibril formation by an SH3 domain. Proc Natl Acad Sci U S A 95:4224–4228.

Hannun YA. 1996. Functions of ceramide in coordinating cellular responses to stress. Science 274:1855–1859.

Iannuzzi C, Maritato R, Irace G, Sirangelo I. 2013a. Misfolding and amyloid aggregation of apomyoglobin. Int J Mol Sci 14:14287–14300.

Iannuzzi C, Maritato R, Irace G, Sirangelo I. 2013b. Glycation accelerates fibrillization of the amyloidogenic W7FW14F apomyoglobin. PLoS One 8: e80768.

Infusini G, Iannuzzi C, Vilasi S, Birolo L, Pagnozzi D, Pucci P, Irace G, Sirangelo I. 2012. Resolution of the effects induced by W/F substitutions on the conformation and dynamics of the amyloid-forming apomyoglobin mutant W7FW14F. Eur Biophys J 41:615–627.

Invernizzi G, Papaleo E, Sabate R, Ventura S. 2012. Protein aggregation: Mechanisms and functional consequences. Int J Biochem Cell Biol 44:1541–1554.

Jomova K, Vondrakova D, Lawson M, Valko M. 2010. Metals, oxidative stress and neurodegenerative disorders. Mol Cell Biochem 345:91–104.

Kadowaki H, Nishitoh H, Urano F, Sadamitsu C, Matsuzawa A, Takeda K, Masutani H, Yodoi J, Urano Y, Nagano T, Ichijo H. 2005. Amyloid β induces

neuronal cell death through ROS mediated ASK1 activation. Cell Death Differ 12:19–24.

Koffie RM, Meyer-Luehmann M, Hashimoto T, Adams KW, Mielke ML, Garcia-Alloza M, Micheva KD, Smith SJ, Kim ML, Lee VM, Hyman BT, Spires-Jones TL. 2009. Oligomeric amyloid beta associates with postsynaptic densities and correlates with excitatory synapse loss near senile plaques. Proc Natl Acad Sci U S A 106:4012–4017.

Kriem B, Sponne I, Fifre A, Malaplate-Armand C, Lozac'h-Pillot K, Koziel V, Yen-Potin FT, Bihain B, Oster T, Olivier JL, Pillot T. 2005. Cytosolic phospholipase A2 mediates neuronal apoptosis induced by soluble oligomers of the amyloid-beta peptide. FASEB J 19:85–87.

Lee T, Malone B, Longobardi L, Balestrieri ML. 2001. Differential regulation of three catalytic activities of platelet-activating factor (PAF)-dependent transacetylase. Arch Biochem Biophys 387:41–46.

Li J, Hu J, Shao B, Zhou W, Cui Y, Dong C, Ezoulin JM, Zhu X, Ding W, Heymans F, Chen H. 2009. Protection of PMS777, a new AChE inhibitor with PAF antagonism, against amyloid-beta-induced neuronal apoptosis and neuroinflammation. Cell Mol Neurobiol 29:589–595.

Litvinovich SV, Brew SA, Aota S, Akiyama SK, Haudenschild C, Ingham KC. 1998. Formation of amyloid like fibrils by self association of a partially unfolded fibronectin type III module. J Mol Biol 280:245–258.

Luheshi LM, Dobson CM. 2009. Bridging the gap: From protein misfolding to protein mis-folding diseases. FEBS Letters 583:2581–2586.

Mattson MP. 1999. Impairment of membrane transport and signal transduction systems by amyloidogenic proteins. Methods Enzymol 309:733–768.

Ortore MG, Spinozzi F, Vilasi S, Sirangelo I, Irace G, Shukla A, Narayanan T, Sinibaldi R, Mariani P. 2011. Time-resolved small-angle X-ray scattering study of the early stage of amyloid formation of an apomyoglobin mutant. Phys Rev E Stat Nonlin Soft Matter Phys 84:061904.

Prescott SM, Zimmerman GA, Stafforini DM, McIntyre TM. 2000. Platelet activating factor and related lipid mediators. Annu Rev Biochem 69:419–445.

Sabirzhanov B, Stoica BA, Hanscom M, Piao CS, Faden AI. 2012. Overexpression of HSP70 attenuates caspase-dependent and caspase-independent pathways and inhibits neuronal apoptosis. J Neurochem 123:542–554.

Sanchez-Mejia RO, Newman JW, Toh S, Yu GQ, Zhou Y, Halabisky B, Cissè M, Scearce-Levie K, Cheng IH, Gan L, Palop JJ, Bonventre JV, Mucke L. 2008. Phospholipase A2 reduction ameliorates cognitive deficits in a mouse model of Alzheimer's disease. Nat Neurosci 11:1311–1318.

Shi C, Wu F, Xu J. 2010. H_2O_2 and PAF mediate Aβ1–42-induced Ca^{2+} dyshomeostasis that is blocked by EGb761. Neurochem Int 56:893–905.

Sirangelo I, Malmo C, Casillo M, Mezzogiorno A, Papa M. 2002. Tryptophanyl substitution in apomyoglobin determine protein aggregation and amyloid-like fibril formation at physiological pH. J Biol Chem 277:45887–45891.

Sirangelo I, Malmo C, Iannuzzi C, Mezzogiorno A, Bianco MR, Papa M, Irace G. 2004. Fibrillogenesis and cytotoxic activity of the amyloid-forming apomyoglobin mutant W7FW14F. J Biol Chem 279:13183–13189.

Sirangelo I, Iannuzzi C, Vilasi S, Irace G, Giuberti G, Misso G, D'Alessandro A, Abbruzzese A, Caraglia M. 2009. W7FW14F apomyoglobin amyloid aggregates-mediated apoptosis is due to oxidative stress and AKT inactivation caused by Ras and Rac. J Cell Physiol 221:412–423.

Sirangelo I, Irace G. 2010. Inhibition of aggregate formation as therapeutic target in protein misfolding diseases: Effect of tetracycline and trehalose. Expert Opin Ther Targets 14:1311–1321.

Sirangelo I, Irace G, Balestrieri ML. 2013. Amyloid toxicity and platelet-activating factor signaling. J Cell Physiol 228:1143–1148.

Tong N, Sanchez JF, Maggirwar SB, Ramirez SH, Guo H, Dewhurst S, Gelbard HA. 2001. Activation of glycogen synthase kinase 3 beta (GSK-3beta) by platelet activating factor mediates migration and cell death in cerebellar granule neurons. Eur J Neurosci 13:1913–1922.

Vilasi S, Iannuzzi C, Portaccio M, Irace G, Sirangelo I. 2008. Effect of trehalose on W7FW14F apomyoglobin and insulin fibrillization: New insight into inhibition activity. Biochemistry 47:1789–1796.

Vilasi S, Sarcina R, Maritato R, De Simone A, Irace G, Sirangelo I. 2011. Heparin induces harmless fibril formation in amyloidogenic W7FW14F apomyoglobin and amyloid aggregation in wild-type protein in vitro. PLoS ONE 6:e22076.

Vilasi S, Sirangelo I, Irace G, Caputo I, Barone MV, Esposito C, Ragone R. 2010. Interaction of "toxic" and "immunogenic" A-gliadin peptides with a membrane-mimetic environment. J Mol Recognit 23:322–328.

Vilasi A, Vilasi S, Romano R, Acernese F, Barone F, Balestrieri ML, Maritato R, Irace G, Sirangelo I. 2013. Unraveling amyloid toxicity pathway in NIH3T3 cells by a combined protomic and 1 H-NMR metabonomic approach. J Cell Physiol 228:1359–1367.

Xu Y, Tao YX. 2004. Involvement of the NMDA receptor/nitric oxide signal pathway in platelet-activating factor-induced neurotoxicity. Neuroreport 15:263–266.