Neurochemistry International 56 (2010) 597–602

Contents lists available at ScienceDirect

Neurochemistry International

journal homepage: www.elsevier.com/locate/neuint

Oxidation of methionine 35 reduces toxicity of the amyloid beta-peptide(1–42) in neuroblastoma cells (IMR-32) via enzyme methionine sulfoxide reductase A expression and function

Francesco Misiti [a,*], M. Elisabetta Clementi [b], Bruno Giardina [b,c]

[a] Department of Health and Motor Sciences, University of Cassino, V.S. Angelo, Polo didattico della Folcara, 03043 Cassino (FR), Italy
[b] CNR Institute of "Chimica del Riconoscimento Molecolare", Largo F. Vito 1, 00168 Rome, Italy
[c] Institute of Biochemistry and Clinical Biochemistry, Catholic University School of Medicine, Largo F. Vito 1, 00168 Rome, Italy

ARTICLE INFO

Article history:
Received 1 November 2009
Received in revised form 22 December 2009
Accepted 5 January 2010
Available online 11 January 2010

Keywords:
Alzheimer's disease
Amyloid β-peptide (Aβ(1–42))
Methionine
Methionine sulfoxide reductase A
ROS

ABSTRACT

The beta amyloid peptide (Aβ), the major protein component of brain senile plaques in Alzheimer's disease, is known to be directly responsible for the production of free radicals that may lead to neurodegeneration. Our recent evidence suggest that the redox state of methionine residue in position 35 (Met-35) of Aβ has the ability to deeply modify peptide's neurotoxic actions. Reversible oxidation of methionine in proteins involving the enzyme methionine sulfoxide reductase type A (MsrA) is postulated to serve a general antioxidant role and a decrease in MsrA has been implicated in Alzheimer's disease. In rat neuroblastoma cells (IMR-32), we used Aβ(1–42), in which the Met-35 is present in the reduced state, with a modified peptide with oxidized Met-35 (Aβ(1–42)Met35^{OX}), as well as an Aβ-derivative in which Met-35 is substituted with norleucine (Aβ(1–42)Nle35) to investigate the relationship between Met-35 redox state, expression and function of MsrA and reactive oxygen species (ROS) generation. The obtained results shown that MsrA activity, as well as mRNA levels, increase in IMR-32 cells treated with Aβ(1–42)Met35^{OX}, differently to that shown by the reduced derivative. The increase in MsrA function and expression was associated with a decline of ROS levels. None of these effects were observed when cells were exposed to Aβ containing oxidized Met35 (Aβ1–42)Met35^{OX}. Taken together, the results of the present study indicate that the differential toxicity of Aβ peptides containing reduced or oxidised Met-35 depends on the ability of the latter form to reduce ROS generation by enhancing MsrA gene expression and function and suggests the therapeutic potential of MsrA in Alzheimer's disease.

1. Introduction

Oxidative stress has been a key topic of research concerning cancer, aging, heart diseases, arthritis, diabetes, and neurodegenerative disorders such as Parkinson's and Alzheimer's diseases (AD) (Butterfield, 2002). Mechanisms proposed for the neurodegeneration in the brain of AD patients generally focus on the beta amyloid peptide (Aβ), a proteolytic product of 40–42 amino acids of the ubiquitously distributed amyloid precursor protein (APP), and its complexes of redox-active metal ions (Butterfield, 2003) and (Bush, 2003). The "Aβ cascade hypothesis" suggests that Aβ aggregates trigger a complex pathological cascade which leads to neurodegeneration in AD (Golde et al., 2006), including generation of H_2O_2 (Behl et al., 1994; Del Rio and Velez-Pardo, 2004; Opazo et al., 2002) and free radical-induced oxidation (Butterfield, 2003; Engelberg, 2004; Sultana et al., 2004) such as lipid peroxidation (Hayashi et al., 2007). Methionine-35 (Met-35) side chain of AβP appears to play a critical role in peptide's neurotoxicity; indeed, this residue is mostly susceptible to oxidation *in vivo* (Vogt, 1995; Butterfield and Bush, 2004), and Aβ bearing oxidized Met-35 is found in considerable amounts in post-mortem AD plaques (Näslund et al., 1994; Kuo et al., 2001). The accumulation of oxidized Met-35 seems to be related to reduced enzymatic reversal of methionine sulfoxide *back* to methionine observed in AD brains (Prasad-Gabbita et al., 1999). In a recent study it has been demonstrated the requirement for Aβ residue Met-35 for oxidative stress in brain of a mammalian model of Alzheimer disease (Butterfield et al., 2010). Methionine sulfoxide reductases catalyze reduction of free and protein-bound methionine sulfoxides to corresponding methionines (Brot et al., 1981; Weissbach et al., 2002). The oxidation of methionine by reactive oxygen species

Abbreviations: AD, Alzheimer's disease; Aβ, amyloid β-peptide; MsrA, methionine sulfoxide reductase A; DMSO, dimethylsulfoxide; HFIP, 1,1,1,3,3,3-hexafluoro-2-propanol; Met35, methionine residue in position 35; met-O, methionine sulfoxide; ROS, reactive oxygen species; DHR, dihydrorhodamine.

* Corresponding author. Tel.: +39 07762994423.
E-mail address: f.misiti@unicas.it (F. Misiti).

0197-0186/$ – see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.neuint.2010.01.002

(ROS) generates a diastereomeric mixture of methionine-S-sulfoxide (Met-S-SO) and methionine-R-sulfoxide (Met-R-SO). Two distinct enzyme families evolved for reduction of these sulfoxides, with methionine-S-sulfoxide reductase (MsrA) being stereospecific for Met-S-SO and methionine-R-sulfoxide reductase (MsrB) for Met-R-SO (Sharov et al., 1999; Moskovitz et al., 2000; Grimaud et al., 2001; Kryukov et al., 2002). Oxidation of selected methionine residues in some proteins, including K+ channels (Ciorba et al., 1997) and calmodulin (Yao et al., 1996), drastically alters their function, suggesting that methionine oxidation and MsrA may have a role in cellular signal transduction (Hoshi and Heinemann, 2001). Methionine oxidation in other proteins, such as glutamine synthetase, however, does not cause any noticeable functional change. This observation led to the speculation that a reversible oxidation–reduction cycle of methionine involving MsrA may also act as a general antioxidant mechanism, functioning as a sink for ROS to protect other cellular components (Levine et al., 1996). The importance of ROS in amyloid-induced cellular injury and the postulated antioxidant potential of MsrA suggests that this enzyme may be involved in the lower toxicity induced by the oxidised derivative of Aβ (Clementi et al., 2005, 2006; Piacentini et al., 2008). We tested this hypothesis by evaluating the effects of Aβ(1–42), in which the Met-35 is present in the reduced state (Aβ(1–42)Met35), were compared to those of a modified peptide with oxidized Met-35 (Aβ(1–42)Met35^{OX}), as well as those of an Aβ-derivative with norleucine substituting Met-35 (Aβ(1–42)Nle35); MsrA expression and activity, as well as ROS generation and methionine sulfoxide levels were investigated in IMR-32 cells after exposure to the above peptides.

2. Materials and methods

2.1. Preparation of Aβ peptides

Amyloid β(1–42) containing oxidized Met35 (Aβ1–42Met35^{OX}) or norleucine in the place of Met35 (Aβ1–42)Nle35 were purchased from Peptide Speciality Laboratories GmbH (Heidelberg, Germany). Analysis of the peptides by reverse-phase high-performance chromatography and mass spectrometry, as supplied by manufacturer, revealed a high degree of purity (>98%). Stock solutions [1 mM in dimethylsulfoxide (DMSO)] were prepared according to the manufacturer's instructions, stored at −80 °C, and thawed and diluted to the final concentration in the proper medium immediately before use. For experiments conducted to determine whether the aggregation of the peptides had any influence on the Aβ-induced effects we observed, we dissolved the Aβ1–42 peptide in 100% 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP, Sigma) (final concentration, 1 mM) to eliminate any aggregate forms that might have been present. The HFIP was then removed by vacuum evaporation, and the remaining film of disaggregated peptide was dissolved in DMSO (Aβ1–42)^{HFIP}, as described above, and used immediately thereafter. In all control experiments, DMSO was added to cell cultures at the same concentrations present in the peptide solutions.

2.2. Cell culture

Human neuroblastoma IMR32 cells were grown in minimum essential medium (Biochrom KG, Berlin, Germany) supplemented with 10% heat-inactivated fetal bovine serum (HyClone, Logan, UT), 100 IU/ml penicillin, and 100 μg/ml streptomycin (Invitrogen, Grand Island, NY, USA). Cells were plated at a concentration of $10^4/cm^2$ in 35-mm-diameter plastic Petri dishes and cultured at 37 °C in an atmosphere of 5% CO_2 in air. Cell differentiation was induced by 1 mM dibutyryl cAMP and 2.5 μM 5-bromodeoxyuridine (Sigma, St. Louis, MO, USA), which were added to the culture medium three times for week, starting from the day after plating. After a week, the differentiated cells were plated at an appropriate density according to each experimental scale.

2.3. Direct toxicity study

Cell survival was evaluated by the 3-[(4,5-dimethylthiazol-2-yl)-5,3-carboxymethoxyphenyl]-2-(4-sulfophenyl)-2H tetrazolium, inner salt (MTS) reduction assay (CellTiter 96 Aqueous One Solution Cell Proliferation Assay, Promega, Madison, WI, USA), as previously described (Clementi et al., 2006). The MTS assay is a sensitive measurement of the normal metabolic status of cells, particularly that of mitochondria, which reflects early cellular redox changes. After exposure to the amyloid peptides (10 μM) for 48 h, cells were treated with the MTS solution (2 mg/ml) and after incubation for 4 h at 37 °C in a 5% CO_2 incubator, the intracellular soluble formazan produced by cellular reduction of the MTS was determined by recording the absorbance of each 96-wells plate using the automatic microplate photometer (SpectraCount – Packard Bioscience Company, Groningen, Netherlands) at a wavelength of 490 nm. The reference wavelenght was 690 nm.

2.4. Reverse transcription and polymerase chain reaction

2.4.1. Total RNA isolate

Total RNA of the cells is extracted using RNA-Bee™ reagent (Biotech, Italy) according to the supplier's instructions. RNA was quantified by optical density measurements at 260 and 280 nm with a spectrophotometer. Integrity was confirmed by running samples on 1% agarose gel.

2.4.2. Synthesis of cDNA

We have used 4 μg of RNA in a 20 μl reaction mixture utilizing M-MLV Reverse Transcriptase Kit (Sigma, St. Louis, MO, USA) according to the supplier's instructions. Resulting reverse transcription products were stored at −20 °C until later use.

2.4.3. Polymerase chain reaction

Human β-actin and MsrA primers were synthesised by invitrogen according to the following sequences (Schallreuter et al., 2006).

MsrA (328 bp): forward 5′-AGTACCTGAGCAAGAACCCCA-3′, reverse 5′-TCACT-CAGACCCCAGAAGACA-3′. β-Actin (606 bp): forward 5′-GAGACCTTCAACACCC-CAGC-3′, reverse 5′-TCTTCATTGTGCTGGGTGCC-3′.

Human β-actin was chosen as internal control. PCR was carried out with Red Taq Polymerase (Sigma, St. Louis, MO, USA) according to the supplier's conditions. The PCR reaction conditions were: 94 °C for 5 min, followed by 35 cycles (30 for β-actin) of 1 min denaturation at 94 °C, 1 min annealing at 54.8 °C, 30 s polymerization at 72 °C and finally 10 min extension at 72 °C. PCR products were analyzed by electrophoresis in agarose 1.8% with ethidium bromide (1 μg/ml) in TBE 1× buffer (Tris 40 mM, EDTA 1 mM, boric acid 44 mM) for 2 h at 80 V (constant voltage) with 123 bp ladder as molecular weight marker.

Images of gels were acquired (Biorad Gel Doc 2000, Hercules, CA, USA) and scanned (Biorad GS800, Hercules, CA, USA) using Biorad Quantity One software. The density of the PCR bands were expressed as a ratio of the band density divided by that of the housekeeping gene, β-actin.

2.5. Methionine reductase system determination

IMR-32 cells were collected and washed once in PBS buffer, resuspended in 300 μl cell lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1 mM EGTA, 1% Triton X-100, 1× Complete® protease inhibitor mix (Roche Diagnostics, Penzberg, Germany)) and mixed thoroughly. After centrifugation (15,000 × g, 10 min, 4 °C), protein concentration of the supernatant was determined using the Coomassie plus reagent (Pierce, Rockford, IL, USA). For activity measurements, a MetO-containing substrate peptide (His_6–Ala–Ala–Gln–MetO–Ile) was incubated with equal amounts of lysates in the presence of 30 mM DTT at 37 °C for 2 h. The peptides were then isolated using NiNTA material (Qiagen) and then analyzed using MALDI-time of flight mass spectrometry as described previously (Jung et al., 2002).

2.6. ROS measurements

The fluorescent dye dihydrorhodamine (DHR) 123 (Calbiochem) was used to estimate the ROS level (Yermolaieva et al., 2000). The excitation and emission wavelengths were 500 and 530 nm, respectively. IMR-32 cells harvested from a confluent flask were resuspended in the standard saline solution (see above), and DHR123 (10 mM stock in DMSO) was added to achieve a final concentration of 10 or 20 μM. To measure the ROS production during amyloid beta peptides treatment, the cells were loaded with DHR123 for 5 min at room temperature, centrifuged, resuspended in a 3-ml cuvette containing the solution that had been used to dissolve amyloid beta peptides, and immediately placed in the spectrofluorometer (FP-750, JASCO, Tokyo, Japan). Because multiple ROS convert DHR123 to the stable fluorescent derivative DHR (Haugland, 1996), the slope of signal at 530 nm during the first 4 min was used to estimate the overall ROS production rate.

2.7. Determination of Met-O levels

The cells were then suspended in 20 mM Tris–HCl pH 7.4 and disrupted by freezing and thawing three times. The cell suspension was centrifuged at 12,000 × g and the supernatant was removed. An aliquot of the supernatant was incubated with 4 μg Pronase for 16 h at 37 °C. The mixture was heated at 100 °C for 1 min and then centrifuged to remove insoluble material. The supernatant was analyzed for its aminoacid composition by a amino acid analyser, as previously reported (Yermolaieva et al., 2004). The two epimers of met-O were the first amino acids to emerge from the column and readily resolve from the other aminoacids. The percentage of met-O present in the samples was calculated as pmol met-O/(pmol methionine + met-O). The estimated values would include any free met-O that could be present in the cytoplasm.

2.8. Statistical analysis

All data are expressed as means ± SEM. Statistical analyses (Student's *t*-test and ANOVA) were performed with SYSTAT 10.2 software (Statcom, Inc., Richmond, CA, USA). The level of significance was set at 0.01.

3. Results

3.1. Effects of Aβ peptides on MsrA gene expression

MsrA mRNA expression was investigated by RT-PCR method using RNA isolated from IMR-32 cells exposed to culture medium or medium containing Aβ peptides. The concentration of Aβ peptides was fixed to 10 μM and gene expression was estimated after 48 h incubation. Specific band for MsrA (Fig. 1) were detected in controls and treated cells. We found that MsrA gene expression was largely up-regulated in the IMR-32 neuroblastoma cells treated with Aβ(1–42)Met35^{OX} peptide compared with the results shown by Aβ(1–42), which was nearly similar to that shown by control cells. Moreover, as expected, Aβ(1–42)Nle35 did not modify the expression of MsrA assessed throughout the experiment.

3.2. Determination of Msr system activity

In order to evaluate Msr system activity in IMR-32 cells, Met-O-reducing activity was determined using Met-O-containing peptides, which were analyzed by MALDI (matrix-assisted laser desorption/ionization) time of flight mass spectrometry before and after 48 h incubation with Aβ peptides (10 μM) (Fig. 2). Compared to the other amyloid beta peptides used, Aβ(1–42)Met35 and Aβ(1–42)Nle35, Msr system activity was strongest in IMR-32 treated with Aβ(1–42)Met35^{OX}. After incubation for 48 h, almost

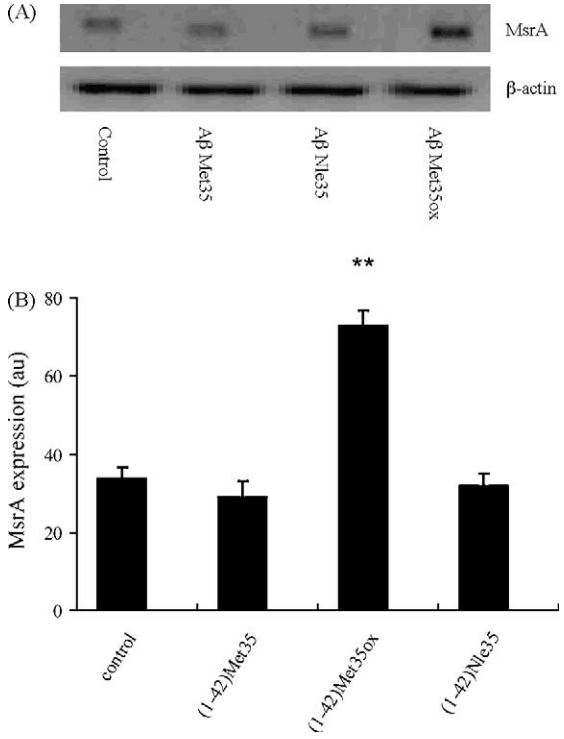

Fig. 1. Panel A: effects of Aβ peptides on MsrA expression. Cells were incubated with medium alone (control) or with medium containing Aβ(1–42)Met35, Aβ(1–42)Met35^{OX} and Aβ(1–42)Nle35 10 μM for 48 h. β-Actin was used as internal control. Panel B: quantification of the intensities of MsrA bands determined by densitometric scanning of agarose gel. Results are from four independent experiments. $^{**}P < 0.001$ vs. control.

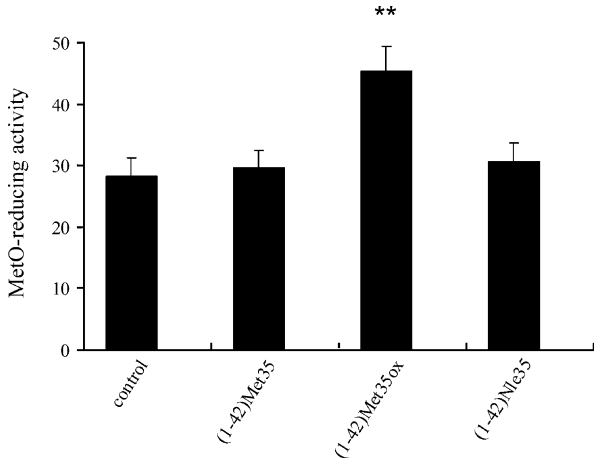

Fig. 2. Effects of Aβ peptides on MsrA enzyme activity. Cells were incubated with medium alone (control) or with medium containing Aβ(1–42)Met35, Aβ(1–42)Met35^{OX} and Aβ(1–42)Nle35 10 μM for 48 h. Msr activity measurements: Met-O containing substrate peptide (His_6–Ala–Ala–Gln–MetO–Ile) was incubated with equal amounts of cell lysates. Peptides were then analyzed using MALDI-TOF mass spectrometry. The histogram shows the relative amount of reduced peptide in the respective experiments. Results are from four independent experiments. $^{**}P < 0.001$ vs. control.

50% of the substrate peptide was reduced by extracts obtained from Aβ(1–42)Met35^{OX} treated cells. Under the same conditions, Aβ(1–42)Met35 and Aβ(1–42)Nle35 treated cells reduced approximately 30%. Additional control experiments were performed with Aβ(1–42)Met35 diluted in DMSO and with the same peptide in HFIP (see Section 2). It has been reported that HFIP pretreatment stabilises Aβ peptides in the monomer form (Berman et al., 2008). The results obtained with these two peptides were not significantly different: Msr system activity, measured after 48 h exposure to Aβ(1–42)^{HFIP} was not significantly different from that obtained with Aβ(1–42)Met35 diluted in DMSO.

3.3. Effects of Aβ peptides on ROS generation

The reversible oxidation–reduction of methionine/met-O involving Msr system has been postulated to serve as a ROS sink (Levine et al., 1996). This idea predicts that up-regulation of MsrA gene expression to facilitate the reduction process should decrease the overall ROS level. As shown in Fig. 3, although Aβ(1–42)Met35 and Aβ(1–42)Met35^{OX} treated cells (10 μM for 48 h) showed ROS levels higher with respect to control, in Aβ(1–42)Met35^{OX} treated cells ROS level were significantly lower with respect to that shown by Aβ(1–42)Met35. This finding is consistent with the prediction that over-expression of MsrA is responsible, almost in part, in reducing the overall ROS level. Moreover, as expected, Aβ(1–42)Nle35 did not modify ROS production assessed throughout the experiment.

3.4. Cellular met-O contents after Aβ exposure

The finding that the mean ROS level was lower in the cells treated with Aβ(1–42)Met35^{OX} suggests that the met-O level in these cells may be also lower. This hypothesis was tested by analyzing the met-O levels in the proteins of the cells treated with amyloid peptides (10 μM for 48 h). There was no significant difference in the level of met-O among the Aβ(1–42)Met35, Aβ(1–42)Met35^{OX} and Aβ(1–42)Nle35 groups when expressed as a percentage of the total methionine. The levels of met-O ranged from 8% to 11%. This finding suggests that the majority of met-O in these cell preparations may be functionally inaccessible to MsrA, as suggested previously (Yermolaieva et al., 2004).

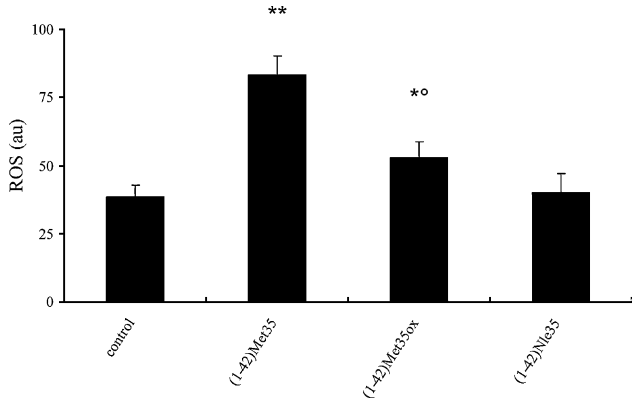

Fig. 3. Effects of Aβ peptides on ROS generation. Cells were incubated with medium alone (control) or with medium containing Aβ(1–42)Met35, Aβ(1–42)Met35^{OX} and Aβ(1–42)Nle35 10 μM for 48 h. The slope of the DHR 123 (20 μM) signal in the first 4 min of the treatment was used to estimate the ROS level. Results are from four independent experiments. $^{**}P < 0.001$ and $^{*}P < 0.01$ vs. control, $^{\circ}P < 0.001$ vs. Aβ(1–42)Met35.

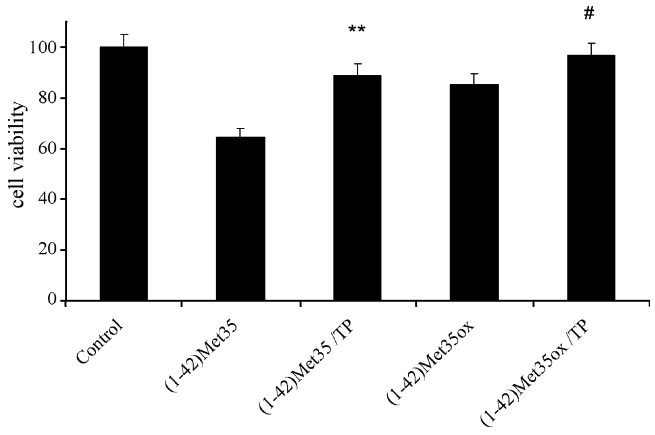

Fig. 4. Inhibitory effects of TEMPOL on Aβ peptide-induced cell death. IMR-32 cell were pre-treated with TEMPOL (TP) 5 mM for 1 h, and then exposed to Aβ(1–42)Met35 and Aβ(1–42)Met35^{OX} 10 μM for 48 h. Cell viability was assessed by MTS assay and data expressed as percent of control. Results are four independent experiments. $^{**}P < 0.001$ vs. Aβ(1–42)Met35, $^{\#}P < 0.01$ vs. Aβ(1–42)Met35^{OX}.

3.5. Effects of TEMPOL on Aβ-induced IMR-32 cell death

To underline the involvement of ROS in the Aβ-induced cell death, the cells were pre-treated for 1 h with a cell-permeable ROS scavenger TEMPOL (5 mM) (Cuzzocrea et al., 2000) prior to Aβ treatment (10 μM). As shown in Fig. 4, cell viability assessed with the MTS assay after 48 h, indicated that pre-treatment with TEMPOL scavenger, significantly increased the percentage of viable cells observed after Aβ(1–42) alone exposure. In contrast, the minor toxic effects of Aβ(1–42)Met35^{OX} were slightly influenced by TEMPOL. These findings suggest that the Met35-dependent toxicity of Aβ in IMR32 neuroblastoma cells is associated with cell death pathways triggered by ROS generation.

4. Discussion

The precise molecular mechanisms responsible for AD-associated neuro-degeneration are not fully understood. However, it has been proposed that Aβ peptide plays a key role in the pathogenesis of the disease (LaFerla, 2002; LaFerla et al., 2007; Mattson, 2007). Aβ1–40 and Aβ1–42, the forms most frequently found in human AD brains, have different biochemical properties, and the 42-residue peptide also displays significantly greater neurotoxicity (Davis and Van Nostrand, 1996; Klein et al., 1999; Dahlgren et al., 2002; Saido and Iwata, 2006; Yun et al., 2007). Several studies indicate that the Met35 residue in the native Aβ plays a key role in peptide's toxicity (Varadarajan et al., 1999; Ciccotosto et al., 2003; Butterfield and Bush, 2004; Clementi et al., 2006; Johansson et al., 2007). The sulfur atom in this residue may be in either the oxidized or reduced state. Aβ peptides containing both forms of Met35 have been isolated in variable amounts from plaques found post-mortem in AD patients' brains (Näslund et al., 1994; Kuo et al., 2001; Butterfield and Bush, 2004). Our recent studies reported that either Aβ(1–42)Met35 and Aβ(1–42)3Met35^{OX}, are able to induce IMR-32 cells degeneration, although Aβ(1–42)Met35 appears to be significantly more potent with respect to the oxidized derivative (Clementi et al., 2006).

In this study, we have shown that the redox state of methionine 35 modulate MsrA expression and activity, thereby protecting neuroblastoma cells from protein oxidative damage and cell death. We had demonstrated that MsrA activity, as well as mRNA levels, increase in IMR-32 cells treated with Aβ(1–42)Met35^{OX}, differently to that shown by the reduced derivative. The increase in the peptide methionine sulfoxide reductase function was associated with a decline of ROS levels. Other studies have reported the involvement of the Msr system in protection against oxidative stress-induced cell death in T lymphocytes (Moskovitz et al., 1998), neuronal PC12 (Yermolaieva et al., 2004) and lens cells (Kantorow et al., 2004).

However, several points regarding Aβ-induced neurotoxicity and the precise role of Msr system in the pathogenesis of AD is not yet clear. And no information is available on the role played by the redox state of Met35 in the reduced enzymatic reversal of methionine sulfoxide *back* to methionine observed in considerable amounts in AD brains (Prasad-Gabbita et al., 1999). These issues were specifically addressed in the present study. Our PCR experiments evidenced that MsrA expression is specifically dependent on the redox state of methionine 35. Unlike Aβ(1–42) and Aβ(1–42)Nle35, Aβ(1–42)Met35^{OX} induced a large increase in MsrA expression. In addition determination of enzymatic activity showed that Aβ(1–42) significantly increase Msr system activity when its Met35 is oxidised to methionine sulfoxide but not when this residue is reduced or replaced with norleucine. Therefore we observed that when IMR-32 cells are challenged with the oxidative derivative of amyloid peptide, a remarkable decrease in ROS levels compared to that shown by other peptides tested was monitored by using the ROS-sensitive intracellular fluorescent dye DHR123 (Yermolaieva et al., 2000), which has been used as a general indicator of cellular ROS production (Royall and Ischiropoulos, 1993; Ischiropoulos et al., 1999). Multiple ROS convert DHR123 into a highly fluorescent form (Haugland, 1996), such that the rate of the fluorescent signal increase reflects the ROS production rate. DHR123 is reported to be more sensitive in detecting ROS than other dyes tested (Vowells et al., 1995).

Many of the surface-exposed methionine residues in the enzyme glutamine synthetase can be oxidized without altering its function. Based on this observation, it was proposed that cyclic oxidation and reduction of methionine residues involving Msr might function as a cellular sink for ROS (Levine et al., 1996). The lower overall ROS level observed in our study in the cell group showing MsrA over-expression is consistent with this idea. The ROS-scavenging hypothesis also predicts that the total met-O level may be higher with greater oxidative stress. However, the total cellular met-O level was not markedly altered by MsrA expression. Thus, there may be a specialized pool of methionine residues that specifically function as a ROS sink. The possibility that repair of specific met-O residues in selected proteins plays a critical role in enhancing the efficacy of other ROS scavenging components cannot be totally ruled out. Our results suggests that

Aβ(1–42)Met35^{OX} induced MsrA up-regulation might prevent accumulation of oxidative damage to proteins and confer an higher resistance than Aβ(1–42)Met35 against oxidative stress-dependent cell death. An enhanced resistance to hydrogen peroxide was shown in human T cells stably transfected with bovine MsrA and exposed to hydrogen peroxide (Moskovitz et al., 1998). Previous our results support our hypothesis, because the differential apoptotic effects elicited by the two peptides on caspase-3 activation, bax/bcl-2 ratio and DNA fragmentation correlate with the difference observed in this study (Clementi et al., 2006; Piacentini et al., 2008). Moreover, as already demonstrated for cysteine oxidation products such as sulfenic and sulfinic acids within proteins (Finkel, 2000, 2003; Poole et al., 2004), oxidized methionines may also be critical components in redox signaling. For example, sulfiredoxin and sestrins that repair cysteine-sulfinic acid in peroxiredoxins are likely important not only for their antioxidant function, but also in signalling pathways sensitive to peroxiredoxin hyperoxidation (Woo et al., 2003; Biteau et al., 2003; Budanov et al., 2004). Similarly, MsrA could modulate signal transduction through the regulation of methionine oxidation/reduction within specific proteins and MsrA over-expression would be expected to impact such redox-sensitive signalling pathways. Intriguingly, in fibroblasts over-expressing MsrA it has been reported a diminution of VDAC-1, the main isotype of VDAC responsible for the increase in outer mitochondrial permeability leading to the release of cytochrome *c* into the cytoplasm during programmed cell death (Tsujimoto and Shimizu, 2000). Accordingly, a low dissipation of the $\Delta\Psi_m$ as well a reduced mitochondrial cytochrome *c* release, have been showed in cells treated with the oxidised derivative of Aβ (Misiti et al., 2004; Clementi et al., 2006).

Our finding that the low ROS levels conferred by up-regulation of MsrA expression in IMR-32 cells correlate with greater cell viability after Aβ treatment is consistent with the idea that oxidative stress is an important factor in cell injury associated with Aβ. The relative importance of ROS generation following Aβ exposure remains to be investigated. This does not exclude the possibility that other mechanisms also contribute to this difference. Recent study also showed that oxidation of Met35 can change Aβ(1–42) conformation thus delaying the formation of oligomers and protofibrils (Johansson et al., 2007; Butterfield and Bush, 2004). Numerous studies have demonstrated correlation between aggregation and toxicity of Aβ peptides showing that fibrillar, protofiblillar, globular and soluble oligomeric, and monomeric form of Aβ(1–42) exert very different effects on neuronal functions and viability (LaFerla et al., 2007; Walsh and Selkoe, 2007; Johansson et al., 2007). Nonetheless, the impact of the Met35 redox status on Aβ-dependent MsrA function alteration observed in our study seems to be largely independent of Aβ aggregation. The results of our experiments seem to suggest that there is no correlation between the capacities for aggregation of the different Aβ peptides we used and their influence on MsrA function. In fact, the effects produced by Aβ(1–42)Met35 dissolved in HFIP prior to its dilution in DMSO were not significantly different to those achieved without HFIP pre-treatment. Recently, we have showed that under our experimental conditions, HFIP pre-treatment dissolves the aggregates which are generated in Aβ(1–42)Met35 solubilised in DMSO solution (Piacentini et al., 2008). Taken together, the results of the present study indicate that, in our experimental model, the differential toxicity of Aβ peptides containing reduced or oxidised Met35 depends on the ability of the latter form to reduce ROS generation by enhancing MsrA gene expression and function. Given the important role played by the oxidative stress in AD (Butterfield, 2002), it is plausible that modulation of the MsrA activity in cells may be beneficial in the treatment of this disease.

Acknowledgements

This work was supported by grants from the Cassino University (FAR, 2007) to F.M.

References

Behl, C., Davis, J.B., Lesley, R., Schubert, D., 1994. Hydrogen peroxide mediates amyloid-β protein toxicity. Cell 77, 817–827.
Berman, D.E., Dall'armi, C., Voronov, S.V., McIntire, L.B., Zhang, H., Moore, A.Z., Staniszewski, A., Arancio, O., Kim, T.W., Di Paolo, G., 2008. Oligomeric amyloid-beta peptide disrupts phosphatidylinositol-4,5-bisphosphate metabolism. Nat. Neurosci. 11, 547–554.
Biteau, B., Labarre, J., Toledano, M.B., 2003. ATP-dependent reduction of cysteine-sulphinic acid by *S. cerevisiae* sulphiredoxin. Nature 425, 980–984.
Brot, N., Weissbach, L., Werth, J., Weissbach, H., 1981. Enzymatic reduction of protein-bound methionine sulfoxide. Proc. Natl. Acad. Sci. U.S.A. 78, 2155–2158.
Budanov, A.V., Sablina, A.A., Feinstein, E., Koonin, E.V., Chumakov, P.M., 2004. Regeneration of peroxiredoxins by p53-regulated sestrins, homologs of bacterial AhpD. Science 304, 596–600.
Bush, A.I., 2003. The metallobiology of Alzheimer's disease. Trends Neurosci. 26, 207–214.
Butterfield, D.A., 2002. Amyloid beta-peptide (1–42)-induced oxidative stress and neurotoxicity: implications for neurodegeneration in Alzheimer's disease brain: a review. Free Radic. Res. 36, 1307–1313.
Butterfield, D.A., 2003. Amyloid β-peptide[1–42]-associated free radical-induced oxidative stress and neurodegeneration in Alzheimer's disease brain: mechanisms and consequences. Curr. Med. Chem. 10, 2651–2659.
Butterfield, D.A., Bush, A.I., 2004. Alzheimer's amyloid beta-peptide (1–42): involvement of methionine residue 35 in the oxidative stress and neurotoxicity properties of this peptide. Neurobiol. Aging 25, 563–568.
Butterfield, D.A., Galvan, V., Bader Lange, M., Tang, H., Sowell, A., Spilman, P., Fombonne, J., Gorostiza, O., Zhang, J., Sultana, R., Bredesen, D.E., 2010. In vivo oxidative stress in brain of Alzheimer disease transgenic mice: Requirement for methionine 35 in amyloid β-peptide of APP. Free Radic. Biol. Med. 48, 136–144.
Ciccotosto, G.D., Barnham, K., Cherny, R.A., Masters, C.L., Bush, A.I., Curtain, C.C., Cappai, R., Tew, D., 2003. Methionine oxidation: implication for the mechanism of toxicity of the amyloid peptide from Alzheimer's disease. Lett. Pept. Sci. 10, 413–417.
Ciorba, M.A., Heinemann, S.H., Weissbach, H., Brot, N., Hoshi, T., 1997. Modulation of potassium channel function by methionine oxidation and reduction. Proc. Natl. Acad. Sci. U.S.A. 94, 9932–9937.
Clementi, M.E., Marini, S., Coletta, M., Orsini, F., Giardina, B., Misiti, F., 2005. Abeta(31–35) and Abeta(25–35) fragments of amyloid beta-protein induce cellular death through apoptotic signals: role of the redox state of methionine-35. FEBS Lett. 579, 2913–2918.
Clementi, M.E., Pezzotti, M., Orsini, F., Sampaolese, B., Mezzogori, D., Grassi, C., Giardina, B., Misiti, F., 2006. Alzheimer's amyloid beta-peptide (1–42) induces cell death in human neuroblastoma via bax/bcl-2 ratio increase: an intriguing role for methionine 35. Biochem. Biophys. Res. Commun. 342, 206–213.
Cuzzocrea, S., McDonald, M.C., Mazzon, E., Siriwardena, D., Costantino, G., Fulia, F., Cucinotta, G., Gitto, E., Cordaro, S., Barberi, I., De Sarro, A., Caputi, A.P., Thiemermann, C., 2000. Effects of tempol, a membrane-permeable radical scavenger, in a gerbil model of brain injury. Brain Res. 875, 96–106.
Dahlgren, K.N., Manelli, A.M., Stine Jr., W.B., Baker, L.K., Krafft, G.A., LaDu, M.J., 2002. Oligomeric and fibrillar species of amyloid-beta peptides differentially affect neuronal viability. J. Biol. Chem. 277, 32046–32053.
Davis, J., Van Nostrand, W.E., 1996. Enhanced pathologic properties of Dutch-type mutant amyloid beta-protein. Proc. Natl. Acad. Sci. U.S.A. 93, 2996–3000.
Del Rio, M.J., Velez-Pardo, C., 2004. The hydrogen peroxide and its importance in Alzheimer's and Parkinson's disease. Curr. Med. Chem. 4, 279–285.
Engelberg, H., 2004. Pathogenic factors in vascular dementia and Alzheimer's disease. Dement. Geriatr. Cogn. Disord. 18, 278–298.
Finkel, T., 2000. Redox-dependent signal transduction. FEBS Lett. 476, 52–54.
Finkel, T., 2003. Oxidant signals and oxidative stress. Curr. Opin. Cell Biol. 15, 247–254.
Golde, T.E., Dickson, D., Hutton, M., 2006. Filling the gaps in the Aβ cascade hypothesis of Alzheimer's disease. Curr. Alzheimer Res. 3, 421–430.
Grimaud, R., Ezraty, B., Mitchell, J.K., Lafitte, D., Briand, C., Derrick, P.J., Barras, F., 2001. Repair of oxidized proteins. Identification of a new methionine sulfoxide reductase. J. Biol. Chem. 276, 48915–48920.
Haugland, R.P., 1996. Handbook of Fluorescent Probes and Research Chemicals. Molecular Probes, Eugene, OR.
Hayashi, T., Shishido, N., Nakayama, K., Nunomura, A., Smith, M.A., Perry, G., Nakamura, M., 2007. Lipid peroxidation and 4-hydroxy-2-nonenal formation by copper ion bound to amyloid-beta peptide. Free Radic. Biol. Med. 43, 1552–1559.
Hoshi, T., Heinemann, S.H., 2001. Regulation of cell function by methionine oxidation and reduction. J. Physiol. (Lond.) 531, 1–11.
Ischiropoulos, H., Gow, A., Thom, S.R., Kooy, N.W., Royall, J.A., Crow, J.P., 1999. Detection of reactive nitrogen species using 2,7-dichlorodihydrofluorescein and dihydrorhodamine 123. Methods Enzymol. 301, 367–373.

Johansson, A.S., Bergquist, J., Volbracht, C., Päiviö, A., Leist, M., Lannfelt, L., Westlind-Danielsson, A., 2007. Attenuated amyloid-beta aggregation and neurotoxicity owing to methionine oxidation. Neuroreport 18, 559–563.

Jung, S., Hansel, A., Kasperczyk, H., Hoshi, T., Heinemann, S.H., 2002. Activity, tissue distribution and site-directed mutagenesis of a human peptide methionine sulfoxide reductase of type B: hCBS1. FEBS Lett. 527, 91–94.

Kantorow, M., Hawse, J.R., Cowell, T.L., Benhamed, S., Pizarro, G.O., Reddy, V.N., Hejtmancik, J.F., 2004. Methionine sulfoxide reductase A is important for lens cell viability and resistance to oxidative stress. Proc. Natl. Acad. Sci. U.S.A. 101, 9654–9659.

Klein, A.M., Kowall, N.W., Ferrante, R.J., 1999. Neurotoxicity and oxidative damage of beta amyloid 1–42 versus beta amyloid 1–40 in the mouse cerebral cortex. Ann. N.Y. Acad. Sci. 893, 314–320.

Kryukov, G.V., Kumar, R.A., Koc, A., Sun, Z., Gladyshev, V.N., 2002. Selenoprotein R is a zinc-containing stereo-specific methionine sulfoxide reductase. Proc. Natl. Acad. Sci. U.S.A. 99, 4245–4250.

Kuo, Y.M., Kokjohn, T.A., Beach, T.G., 2001. Comparative analysis of amyloid-beta chemical structure and amyloid plaque morphology of transgenic mouse and Alzheimer's disease brains. J. Biol. Chem. 276, 12991–12998.

LaFerla, F.M., 2002. Calcium dyshomeostasis and intracellular signalling in Alzheimer's disease. Nat. Rev. Neurosci. 3, 862–872.

LaFerla, F.M., Green, K.N., Oddo, S., 2007. Intracellular amyloid-beta in Alzheimer's disease. Nat. Rev. Neurosci. 8, 499–509.

Levine, R.L., Mosoni, L., Berlett, B.S., Stadtman, E.R., 1996. Methionine residues as endogenous antioxidants in proteins. Proc. Natl. Acad. Sci. U.S.A. 93 (26), 15036–15040.

Mattson, M.P., 2007. Calcium and neurodegeneration. Aging Cell 6, 337–435.

Misiti, F., Martorana, G.E., Nocca, G., Di Stasio, E., Giardina, B., Clementi, M.E., 2004. Methionine 35 oxidation reduces toxic and pro-apoptotic effects of the amyloid beta-protein fragment (31–35) on isolated brain mitochondria. Neuroscience 126, 297–303.

Moskovitz, J., Flescher, E., Berlett, B.S., Azare, J., Poston, J.M., Stadtman, E.R., 1998. Overexpression of peptide-methionine sulfoxide reductase in Saccharomyces cerevisiae and human T cells provides them with high resistance to oxidative stress. Proc. Natl. Acad. Sci. U.S.A. 95, 14071–14075.

Moskovitz, J., Poston, J.M., Berlett, B.S., Nosworthy, N.J., Szczepanowski, R., Stadtman, E.R., 2000. Identification and characterization of a putative active site for peptide methionine sulfoxide reductase (MsrA) and its substrate stereospecificity. J. Biol. Chem. 275, 14167–14172.

Näslund, J., Schierhorn, A., Hellman, U., Lannfelt, L., Roses, A.D., Tjernberg, L.O., Silberring, J., Gandy, S.E., Winblad, B., Greengard, P., 1994. Relative abundance of Alzheimer Aβ amyloid peptide variants in Alzheimer disease and normal aging. Proc. Natl. Acad. Sci. U.S.A. 91, 8378–8382.

Opazo, C., Huang, X., Cherny, R.A., Moir, R.D., Roher, A.E., White, A.R., Cappai, R., Masters, C.L., Tanzi, R.E., Inestrosa, N.C., Bush, A.I., 2002. Metalloenzyme-like activity of Alzheimer's disease β-amyloid—Cu-dependent catalytic conversion of dopamine, cholesterol, and biological reducing agents to neurotoxic H_2O_2. J. Biol. Chem. 277, 40302–40308.

Piacentini, R., Ripoli, C., Leone, L., Misiti, F., Clementi, M.E., D'Ascenzo, M., Giardina, B., Azzena, G.B., Grassi, C., 2008. Role of methionine 35 in the intracellular Ca^{2+} homeostasis dysregulation and Ca^{2+}-dependent apoptosis induced by amyloid beta-peptide in human neuroblastoma IMR32 cells. J. Neurochem. 107, 1070–1082.

Poole, L.B., Karplus, P.A., Claiborne, A., 2004. Protein sulfenic acids in redox signaling. Annu. Rev. Pharmacol. Toxicol. 44, 325–347.

Prasad-Gabbita, S., Aksenov, M.Y., Lovell, M.A., Markesbery, W.R., 1999. Decrease in peptide methionine sulfoxide reductase in Alzheimer's disease brain. J. Neurochem. 73, 1660–1666.

Royall, J.A., Ischiropoulos, H., 1993. Evaluation of 2′,7′-dichlorofluorescin and dihydrorhodamine 123 as fluorescent probes for intracellular H_2O_2 in cultured endothelial cells. Arch. Biochem. Biophys. 302, 348–355.

Saido, T.C., Iwata, N., 2006. Metabolism of amyloid beta peptide and pathogenesis of Alzheimer's disease. Towards presymptomatic diagnosis, prevention and therapy. Neurosci. Res. 54, 235–253.

Schallreuter, K.U., Rübsam, K., Chavan, B., Zothner, C., Gillbro, J.M., Spencer, J.D., Wood, J.M., 2006. Functioning methionine sulfoxide reductases A and B are present in human epidermal melanocytes in the cytosol and in the nucleus. Biochem. Biophys. Res. Commun. 342, 145–152.

Sharov, V.S., Ferrington, D.A., Squier, T.C., Schoneich, C., 1999. Diastereoselective reduction of protein-bound methionine sulfoxide by methionine sulfoxide reductase. FEBS Lett. 455, 247–250.

Sultana, R., Newman, S., Mohmmad-Abdul, H., Keller, J.N., Butterfield, D.A., 2004. Protective effect of the xanthate, D609, on Alzheimer's amyloid β-peptide (1–42)-induced oxidative stress in primary neuronal cells. Free Radic. Res. 38, 449–458.

Tsujimoto, Y., Shimizu, S., 2000. VDAC regulation by the Bcl-2 family of proteins. Cell Death Differ. 7, 1174–1181.

Varadarajan, S., Yatin, S., Kanski, J., Jahanshahi, F., Butterfield, D.A., 1999. Methionine residue 35 is important in amyloid beta-peptide-associated free radical oxidative stress. Brain Res. Bull. 50, 133–141.

Vogt, W., 1995. Oxidation of methionyl residues in proteins: tools, targets, and reversal. Free Radic. Biol. Med. 18, 93–105.

Vowells, S.J., Sekhsaria, S., Malech, H.L., Shalit, M., Fleisher, T.A., 1995. Flow cytometric analysis of the granulocyte respiratory burst: a comparison study of fluorescent probes. J. Immunol. Methods 178, 89–97.

Walsh, D.M., Selkoe, D.J., 2007. A beta oligomers—a decade of discovery. J. Neurochem. 101, 1172–1184.

Weissbach, H., Etienne, F., Hoshi, T., Heinemann, S.H., Lowther, W.T., Matthews, B., St John, G., Nathan, C., Brot, N., 2002. Peptide methionine sulfoxide reductase: structure, mechanism of action, and biological function. Arch. Biochem. Biophys. 397, 172–178.

Woo, H.A., Chae, H.Z., Hwang, S.C., Yang, K.S., Kang, S.W., Kim, K., Rhee, S.G., 2003. Reversing the inactivation of peroxiredoxins caused by cysteine sulfinic acid formation. Science 300, 653–656.

Yao, Y., Yin, D., Jas, G.S., Kuczer, K., Williams, T.D., Schoneich, C., Squier, T.C., 1996. Oxidative modification of a carboxyl-terminal vicinal methionine in calmodulin by hydrogen peroxide inhibits calmodulin-dependent activation of the plasma membrane Ca-ATPase. Biochemistry 35, 2767–2787.

Yermolaieva, O., Brot, N., Weissbach, H., Heinemann, S.H., Hoshi, T., 2000. Reactive oxygen species and nitric oxide mediate plasticity of neuronal calcium signalling. Proc. Natl. Acad. Sci. U.S.A. 97, 448–453.

Yermolaieva, O., Xu, R., Schinstock, C., Brot, N., Weissbach, H., Heinemann, S.H., Hoshi, T., 2004. Methionine sulfoxide reductase A protects neuronal cells against brief hypoxia/reoxygenation. Proc. Natl. Acad. Sci. U.S.A. 101, 1159–1164.

Yun, S., Urbanc, B., Cruz, L., Bitan, G., Teplow, D.B., Stanley, H.E., 2007. Role of electrostatic interactions in amyloid beta-protein (A beta) oligomer formation: a discrete molecular dynamics study. Biophys. J. 92, 4064–4077.