Methylglyoxal reduces mitochondrial potential and activates Bax and caspase-3 in neurons: implications for Alzheimer's Disease

Marta Tajes[a], Abel Eraso-Pichot[a], Fanny Rubio-Moscardó[a], Biuse Guivernau[a], Mònica Bosch-Morató[a], Victòria Valls-Comamala[a], Gian Pietro Miscione[b,c], Jordi Villà-Freixa[b,d], Toshiharu Suzuki[e], and Francisco J. Muñoz[a *]

[a]Laboratory of Molecular Physiology and Channelopathies, Departament de Ciències Experimentals i de la Salut (DCEXS), Universitat Pompeu Fabra (UPF), Barcelona, Spain

[b]Computational Biochemistry and Biophysics Laboratory, Research Program on Biomedical Informatics, DCEXS, IMIM/UPF, Barcelona

[c]Dipartimento di Chimica "G. Ciamician", Università degli Studi di Bologna, Italy

[e] Escola Politècnica Superior, Universitat de Vic, Spain

[d] Laboratory of Neuroscience, Graduate School of Pharmaceutical Sciences, Hokkaido University, Sapporo, Japan

* To whom correspondence should be addressed: Dr. Francisco J. Muñoz, Lab. Fisiologia Molecular i Canalopaties, Departament de Ciències Experimentals i de la Salut, Universitat Pompeu Fabra, C/ Dr. Aiguader 88, Barcelona 08003, Spain; Tel: +34 93 316 08 52; Fax: +34 93 316 09 01; e-mail: paco.munoz@upf.edu

Abstract

Nitrosative stress has been shown to be responsible for the nitrotyrosination of several proteins. The nitration of triosephosphate isomerase (TPI), a glycolytic enzyme, has been proposed as the link between the extracellular Aß-induced oxidative stress and the intracellular tau protein aggregation in Alzheimer disease. TPI catalyzes the interconversion between glyceraldehyde 3-phosphate (GAP) and dihydroxyacetonphosphate (DHAP). Methylglyoxal (MG) is a toxic side product of the malfunction of the enzyme, and it has been involved in a harmful glycation process. We found an increased nitrotyrosination of TPI induced by Aß in neuroblastoma cells, and a consequent increase in MG production. Aß and MG toxicity triggered the decrease of mitochondrial transmembrane potential, one of the first apoptotic events. Therefore, focusing on the toxic role of MG we described the proapoptotic effects of MG, such as decreasing the protective Bcl2 and increasing the proapoptotic caspase-3 and Bax. Moreover, we used two TPI mutants (Y165F and Y209F) to mimic nitrosative modifications due to Aβ action. Neuroblastoma cells transfected with TPI mutants consistently triggered an increase in the MG production. It is shown that Aß-induced nitration of the two tyrosines closed to the catalytic center of TPI is importantly involved in MG production and this toxic by-product is playing a key role in the neuronal death induced by Aβ oligomers.

Keywords: Alzheimer disease; 3- nitrotyrosine; amyloid; triose-phosphate isomerase; apoptosis; methylglyoxal

The abbreviations used are: Ab, Antibody; Aß, Amyloid ß-peptide; AD, Alzheimer disease; AGEs, advanced glycation end-products; DHAP, dihydroxyacetone phosphate; FBS, fetal bovine serum; GAP, D-glyceraldehyde 3-phosphate; H_2O_2, hydrogen peroxide; MG, methylglyoxal; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NO, nitric oxide; nNOS, neuronal NO synthase; o.e., overexpressing; TPI, triosephosphate isomerase; WT, Wild type**.**

Highlights

(3 de 85 characters maxim)

- Aß induces TPI nitrotyrosination increasing methylglyoxal production

- Methylglyoxal Aß-induced triggers cellular apoptosis

- TPI and methylglyoxal are involved in Aß toxicity

1. Introduction

Nitration of tyrosine is a pathological event associated with several neurodegenerative diseases; and in particular is increased in AD patients (Smith et al, 1997). Amyloid ß peptide (Aß) reduce Cu2 and Fe2 to generate H2O2 and downstream highly reactive ROS (Radical Oxigen Species) (Cherny et al., 2001; Dikalov et al., 2004; Hu et al., 2006; Nelson and Alkon, 2005; Opazo et al., 2002; Tabner et al. 2002) (Greenough et al., 2012). This ROS, among them superoxide anion ($O_2^{\cdot -}$), and nitric oxide (NO), whose production is altered in AD(6-7 Freeradical), react to form the highly reactive peroxynitrite anion(ONOO-) (Castegna et al, 2003; Guix et al, 2005) involved in protein nitrotyrosination. This modification is an irreversible reaction that adds a nitro group (NO_2) to a tyrosine residue, generating 3-nitrotyrosine [9,10free]. One of the proteins more nitrotyrosinated in AD is the glycolytic enzyme triosephosphate isomerase (TPI) (Coma et al. 2005, 15-16free). TPI is a key enzyme in cell metabolism that controls the glycolytic flow and energy production catalyzing the interconversion of D-glyceraldehyde-3-phosphate (GAP) to dihydroxyacetone phosphate (DHAP) (Richard, 1993). Any interruption of glycolysis causes brain dysfunction and memory loss, favoring neurodegeneration [18]. TPI is the only glycolytic enzyme whose functional deficiency is associated with neurodegeneration [19] and has been related to reduced longevity [20]. Moreover, TPI deficiency leads to the accumulation of its substrate DHAP, which can decompose non-enzymatically into methylglyoxal (MG), a cytotoxic precursor of advanced glycation end-products (AGEs) [21].

The present work showed the importance of TPI nitrotyrosination in AD, proposing MG production as the mechanism behind one of deleterious

consequences of Aβ toxicity. We compared the toxic effects of treating neuroblastoma cells (SH-SY5Y) with $A\beta_{1-42}$ oligomers and with MG, and we deepen in apoptosis induced by the toxic glycolytic by-product. In addition, we studied the effect of Tyr165 and Tyr209 nitration (the two tyrosines located close to the catalytic center of TPI) using the Phe mutation of Tyr that mimics the nitro-TPI structure.

2. Materials and Methods

2.1.Aß$_{1-42}$ oligomer preparation

Synthetic $A\beta_{1-42}$ (EZBiolab, Carmel, USA) oligomers were obtained by dissolving 300 µg freeze-dried aliquots in 20 µL DMSO. Peptide stock aliquots were diluted in 0.1 M Tris-HCl at pH 7.4 to a final concentration of 88.6 µM Aβ. Solutions were stirred continuously at 37ºC and 300rpm for 3 h and kept at -80 ºC before being used

2.2. Aß$_{1-42}$ oligomer incubation with human neuroblastoma cells

SH-SY5Y, a human neuroblastoma cell line (supplied by ECACC), was grown in DMEM (Invitrogen) supplemented with 15% FBS and 1% streptomycin/penicillin at 37ºC in a humidified atmosphere containing 5% CO2. For cell viability and metabolism assays, cells were seeded into 96-well culture plates at a density of 25,000 cells / 200 µL per well. For Western blot analysis, cells were plated onto 60mm-diameter-dishes at a density of 700,000 cells / 3 mL per dish.

After 24 h in culture, cells were treated with different concentrations of Aβ oligomers (1 μM) and MG (100, 250 and 500 μM) (Sigma) for another 24 h. Then the cells were lysed on ice with a solution containing 1 M Tris-HCl, 1% Nonidet P-40, 150 mM NaCl, 5 mM EDTA, 1 mM sodium orthovanadate, 1 mM dithiotreitol, pH 7.4, and a protease inhibitor cocktail. Protein concentration was determined by Bradford assay.

2.3. TPI immunoprecipitation

200 μg of total protein from SH-SY5Y cells lysates were incubated with 1.25 μg of anti-TPI polyclonal antibody (Ab) o.n. at 4ºC. Protein G immobilized on sepharose was added and samples were shaken for 2 h at RT. Aggregates were pulled down by centrifugation at 10,000 rpm for 10 min and washed thrice. Protein G and Ab were removed from the immunoprecipitated proteins by boiling the samples for 6 min at 100 ºC.

2.4. Western blot

For analysis of cell homogenate aliquots, gels were transferred to PVDF membranes and incubated for 3 h at room temperature with one of 7 solutions: mouse anti-argpyrimidine monoclonal Ab (1:1,000; Cosmo Bio Co., Ltd), mouse anti-nitrotyrosine monoclonal Ab (1:1,000; Cayman Chemical, Michigan, USA), rabbit anti-cleaved caspase-3 (Asp175) Ab, rabbit anti-Bax Ab (1:1,000; Cell Signaling, Beverly, USA), mouse-anti TPI Ab (1:1000; Abcam), rabbit anti Bcl-2 Ab (1:1000; Cell Signaling), or mouse anti-β-actin monoclonal Ab (1:5,000, Sigma, St. Louis, USA). Membranes were incubated with peroxidase-conjugated secondary Ab (1:3,000; GE-Healthcare, UK) for 1 h at RT. Bands

were visualized using the enhanced chemiluminescence substrate (Super Signal; Pierce).

2.5. Immunocytochemistry of SH-SY5Y

After 24h, cells were fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton X-100. SH-SY5Y cells were immunostained with 1:100 mouse monoclonal anti-methylglyoxal Ab (Cosmo Bio Co., Ltd.) and 1:2,000 Alexa 555-bound as secondary Ab (Sigma) at RT. Transfected cells were positive for EGFP fluorescence. Coverslips were mounted and analyzed using a Leica TCS SP confocal microscope and analyzed with Leica confocal software.

2.6. Measurement of $\Delta\Psi_m$

SH-SY5Y was re-suspended at 10^6 cells/mL in PBS with the fluorophore MitoTracker Red CMXRos (Molecular Probes, Carlsbad, USA). Cells were incubated for 10 min at 37ºC and analyzed in a FACScan (Becton Dickinson, CA).

2.7. Cell viability assays

SH-SY5Y cells were seeded in 96-well plates at a density of 25,000 cells / 200 µL, incubated 24 h, and tested for viability by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction. Briefly, 11 µL of MTT stock solution (5mg/mL) were added; after 2h the reaction was stopped with 120 µL of DMSO. MTT reduction was determined in a plate reader spectrophotometer at 540 and 650 nm. Control cells were taken as 100%.

2.8. Overexpression of TPI in neuroblastoma cells

TPI was amplified by PCR from purified human chromosomal DNA and cloned into a construct containing a 5' upstream flag sequence. Wild-type (WT) TPI and TPI carrying a tyrosine mutation (Y165F or Y209F) were produced and subcloned into a pcDNA3 plasmid. A human neuroblastoma cell line (SH-SY5Y) was seeded in 6-well plates at a density of 5 x 10^5 cells per well and grown for 24 h with Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Afterwards, 2 µg per well of each construct was transfected using Lipofectamine 2000 Reagent (Invitrogen, Carlsbad, USA), following manufacturer instructions. After 3 h, the medium was replaced by DMEM plus 10% FBS and 1% penicillin/streptomycin.

2.9. Statistical analysis

Data were expressed as the mean ± SEM of the values from the number of experiments as indicated in the corresponding figures. Continuous data were evaluated by Student *t*-test.

3. Results

3.1. Aß induces TPI nitrotyrosination

TPI has been demonstrated to be nitrotyrosinated in AD brains (30, 55). The specificity of TPI nitrotyrosination in AD was demonstrated when neuronal cells were challenged with Aß oligomers for 24 hours (Figure 1A) and the immunoprecipitated TPI was strongly positive for nitrotyrosination in treated cells compared with untreated control cells. Furthermore, to analyse the

increase in methylglyoxal production due to Aß, we challenged again neuroblastoma cells with Aß oligomers, and demonstrated by immunofluorescence techniques the increase of glycation due to the toxic peptide (Figure 1B). The characteristic memory loss and dementia in AD are caused by Aβ-induced neurodegeneration. Thus we addressed a comparative study of the effects of Aβ oligomers and MG on apoptosis in human neuroblastoma cells. We focused on one of the early intracellular events that occur in apoptosis, the abnormal opening of the mitochondrial transition pore by the collapse of mitochondrial transmembrane potential ($\Delta\Psi_m$), which results in a rapid release of caspase activators [33]. The challenge with both Aβ oligomers and MG induced a significant increase in the number of cells with diminished $\Delta\Psi_m$ compared to controls (Fig. 1C,D).

3.2. MG treatments induce apoptosis in human neuroblastoma cells

Once we showed the Aß-induced nitrotyrosination of TPI triggered an increase in MG production, we studied cell viability after 24h challenged with increasing concentrations of MG (Fig. 2A) to deepen in the toxicity of increase levels of MG induced by Aß. MG was assayed at different concentrations (Fig. 2A), looking for subtoxic concentrations to establish the lowest threshold triggering cell death and neurotoxicity. We found toxic effects at 250 μM MG, similar to that reported in the literature [32] . Although 250 μM MG is a higher concentration than is expected under physiological conditions [31] it mimics the MG production maintained by nitrotyrosinated TPI throughout the 24-hour Aβ challenge. We observed that MG-induced cell death was dose-dependent.

After a decrease in $\Delta\Psi_m$ there is an abnormal opening of the mitochondrial transition pore due to its activation by pro-apoptotic effectors, with Bax being one of the most important. Bax levels were significantly increased when human neuroblastoma cells were challenged with MG (Fig. 2B). On the other hand, analysis of the protective anti-apoptotic protein Bcl-2 levels (Fig. 2B) showed that neuroblastoma cells had significantly decreased levels of Bcl-2 when challenged by MG, making them more prone to apoptosis.

Caspase-3 is down-stream of the activation of intracellular apoptotic pathways and has been shown to be elevated in neurons and astrocytes of AD patients [34]. As expected after the proapoptotic changes we observed in mitochondria, MG significantly increased the levels of active caspase-3 compared to non-treated human neuroblastoma cells (Fig. 2C).

3.3. Mutations of TPI at Y209F and Y165F induce MG production

We have shown, as previous reports, that TPI nitrotyrosination induces an increased MG production (30). In order to mimic the effect of the nitrotyrosination of the two Tyrosines (Tyr165 and Tyr209) involved in H-bonds characteristic of the open and closed form of the loop respectively, we have replaced them with Phenylalanines (brain xxx). Consistently, we have observed that there is an augmented production of MG in TPI mutants (Figure 3A,B), as we analyzed by the identification of glycation products (argpyrimidines). Interestingly we have found that TPI mutations Y209F and Y165F induce its own glycation (Figure 3C). The physiopathological role of this effect could be related with an increased alteration of TPI function.

4. Discussion

TPI functional deficiency has been associated to neurodegeneration (XX). Furthermore, inefficient glycolysis and ATP reduction are characteristic in AD, so understanding the malfunction of this enzyme is a central issue for many biochemical studies (14, 15, 20, 22, 23, 30–32). Figure S1 shows a schema of the effect of nitrotyrosination on the function of TPI. Under some pathological conditions, like inflammation, defective mithocondrial respiration or oxydative stress, an overproduction of nitric oxide (NO) and superoxide (O_2^-) can take place in the cell. Nitric oxide and superoxide can react and yield peroxynitrite $ONOO^-$, which is a strong nitrating agent and can introduce a nitro group into the Tyrosine phenolic ring, which is strongly activated by the ortho-para directing hydroxyl group, and give rise to 3- NitroTyrosine (NTyr)(35– 40). There is an increasing number of data suggesting that the levels of NTyr in proteins can be considered as a footprint of nitro-oxidative damage, which correlates with protein and enzyme functional alteration and consequent disease development (35, 41–50). When tyrosines are nitrated, a significant decrease in the enzymatic activity is detected: kcat /Km is reduced by 15-fold with respect to the control TPI (30).

The TPI monomer has four tyrosines, two of them, Tyr165 and Tyr209, interact directly, sit very close to the catalytic site, and contribute to loop 6 mobility. Loop 6 is very flexible and plays a key role in the reactivity of the enzyme [40]. We selected Tyr165 and Tyr209 for study because of their importance to enzyme efficiency and their role in MG generation.

MG is the main dicarbonyl compound involved in the AGEs (Advanced glycation endproducts) formation (Thornalley, 2005), which can induce apoptosis by activating numerous intracellular signal transduction pathways (Yamagishi et al., 2002). Formation and accumulation of AGEs has been proposed to be involved in the evolution of AD (33X-H Li, Zhou, glycation exacerbates the neuronal toxicity, cell death and disease), and its levels are increase in aging (21..Konova E, ..Velkova, age-related changes in the glc 2004). AGEs are increased in the AD brains and the glycated Aß accumulation accelerates Aß deposition (5,34). Even, it is showed the higher toxicity of glycated Aß (Aß+ MG) than the normal one (Li et al, 2013). Higher levels of plasma MG were associated with a faster rate of cognitive decline (Beeri MS, Moshier,.. Silverman, jm 2011 serum concentration of an infl). Furthermore, high levels of MG have been found in the cerebrospinal fluid of AD patients and in plasma of diabetic individuals (Kuhla et al, 2005; McLellan et al., 1994; Huang 2012---) which had shown that the MG levels in CSF of AD patients were twofold higher than in controls, and were five to seven times higher than in plasma. MG can modify amino acids, nucleic acids and proteins, and arginine appears to be a primary target in protein modification triggering the formation of argpyrimidine (9-11 Junghyun Kim, ..Jin Sook Kim, BBRC 2010).

Our results confirm the increased nitrotyrosination of TPI due to the Aß effect, and the subsequent increased in methylglyoxal formation. Mitochondria play an essential role in cell death because the permeability of the transition pore and the collapse of $\Delta\Psi_m$ are the initial stages in intracellular apoptotic signalling [34]. And we showed that both molecules could induce this primary

apoptotic event. In fact there are studies suggesting that methylglyoxal could be the link between cognitive dysfunction and diabetes (Xiaobo Huang, ...Kristine von Maltzan, 2012, Possible link between cognitive dysfunc)).

These data suggest that TPI nitrotyrosination plays a key role to induce the neurodegeneration triggered by Aβ. These results confirm existing evidence that apoptosis is the underlying mechanism for the neurodegeneration caused by TPI nitrotyrosination, reported in AD (39;44;[46]) and in Aβ-induced nitro-oxidative stress (37; [47]. We also described a complete apoptosis induction due to the treatment of neuroblastoma cells with MG, showing an increase in the pro- apoptotic Bax protein and the active caspase-3, and a decrease of the anti-apoptotic Bcl-2. Caspase-3 is critical in apoptosis onset and a recent study links it to enhanced synaptic failure in AD, a very early event in the disease onset [28].

5. Discussion

In summary, we have showed that one of the Aß oligomers toxic effects is the nitrotyrosination of TPI, and the consequent increase on MG levels. This toxic by-product can explain part of the apoptosis triggered by Aß oligomers. Furthermore we have shown the importance on nitrotyrosination of the two tyrosines closed to the catalytic center of the glycolytic enzyme through Aß nitrosative stress. This finding explains part of not fully understood Aß oligomers toxicity through methylglyoxal effect.

Acknowledgements

This work was supported by the Plan Estatal de I+D+i 2013-2016 and the ISCIII-Subdirección General de Evaluación y Fomento de la Investigación (Grants PI13/00408, and Red HERACLES RD12/0042/0014) and FEDER Funds; the virtual physiological human (VPH) NoE (FP7-ICT-2007-2-223920), the Spanish Ministry of Science and Innovation (CTQ2008-00755; BFU2006-28430-E/BMC and RETIC COMBIOMED RD07/0067/0001); Generalitat de Catalunya (AGAUR BE-2 10240); and La Marató de TV3 (Nº 100310).

References

Figure/Table Legends

Fig.1 Increased TPI nitrotyrosination and MG levels due to Aß toxicity**. (**A) SH-SY5Y cells were treated with 1 µM Aß for 24 hrs and Western blot studies were performed to demonstrate the nitrotyrosination of TPI by Aß action. (B) Neuroblastoma cells treated with 1 µM Ab oligomers for 24 hrs and Immunocitochemistry image were performed to demonstrate the increase of methylglyoxal production. **(**C**)** Representative graph of ΔΨm in cells challenged with 1 µM $A\beta_{1-42}$ oligomers and 500 µM MG. Assayed by flow cytometry and MitoTracker staining. (D) Quantification of cells with low ΔΨm expressed as percentage regarding control. Data are mean ± SEM of 3 independent experiments performed in triplicate. * $p<0.05$ *vs* control by Student t test.

Fig. 2 Decreased cell viability in neuroblastoma cells challenged with MG. (A) Cells challenged with increasing concentrations of MG, assayed by MTT reduction. Data are mean ± SEM of 6 independent experiments performed in triplicate. ** $p<0.01$, *** $p<0.001$ *vs* control by Student t test. Enhancement of intracellular apoptotic pathway by MG in neuroblastoma cells. (B) Western blot analysis of pro-apoptotic Bax and anti-apoptotic Bcl-2 levels in cells challenged with increasing concentrations MG. Quantifications expressed as AU are shown in the graphs. Data are mean ± SEM of 6 independent experiments.* $p<0.05$, ** $p<0.01$, *** $p<0.001$ *vs* control by Student t test. (C) Western blot analysis of activated caspase-3 levels in cells treated MG at different concentrations. It was

quantified and expressed as AU. Data are mean ± SEM of 6 independent experiments.* $p<0.05$, ** $p<0.01$, *** $p<0.001$ by Student t test.

Fig. 3 Protein glycation colocalizes with TPI mutated at Y165F/Y209F SH-SY5Y cells were transfected with wild type TPI and TPI carrying single mutations (Y165F and Y209F) searching for an increased methylglyoxal production and the consequent protein glycation. (A) Cells were incubated with an anti-argpyrimidine Ab (red staining) to demonstrate the action of MG on proteins. Transfected cells were EGFP positive (green staining). Images were analyzed by confocal microscopy. pCDNA3 was used as the transfection control. (B) Transfected cells were grown for 24 h. After lysis, western blot analysis was carried out to demonstrate the presence of glycated proteins. (C) Glycation was quantified and expressed as A.U. Data are mean SEM of 3 independent experiments. * $p<0.05$ by Student t test. (D) TPI from transfected cells was analyzed for glycation by Western blot analysis with an anti-argpyrimidine Ab. (E) TPI glycation was quantified and expressed as A.U. Data are mean SEM of 3 independent experiments. *$p<0.05$ by Student t test.

Figures/Tables

Figure1

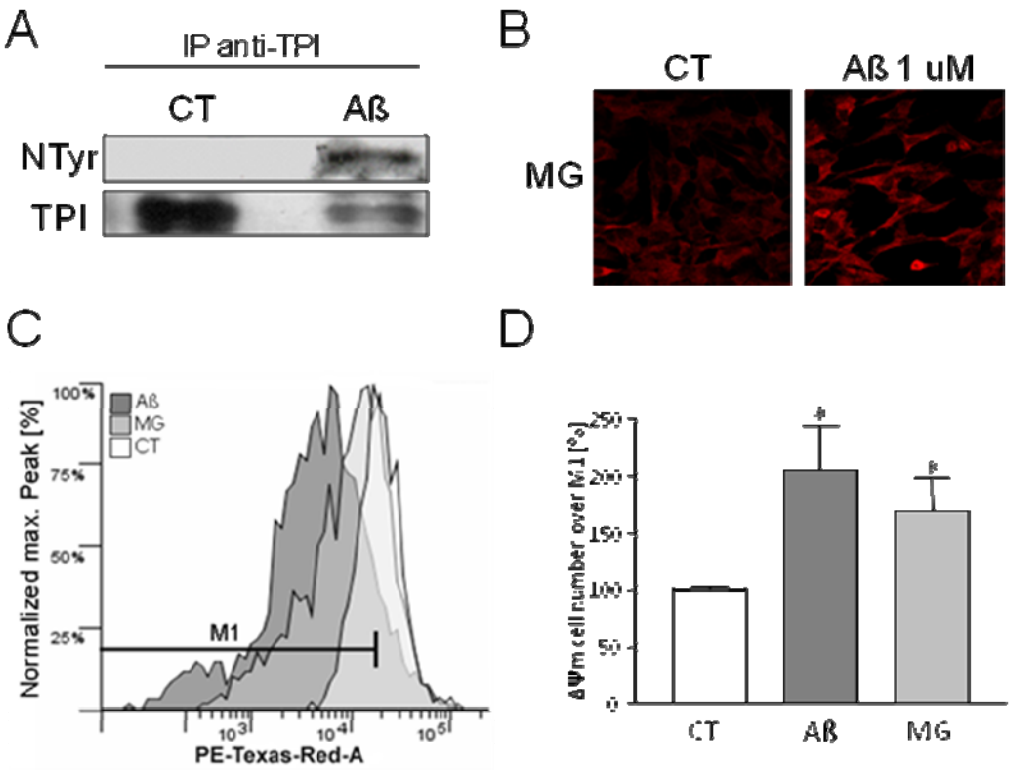


Figure 2

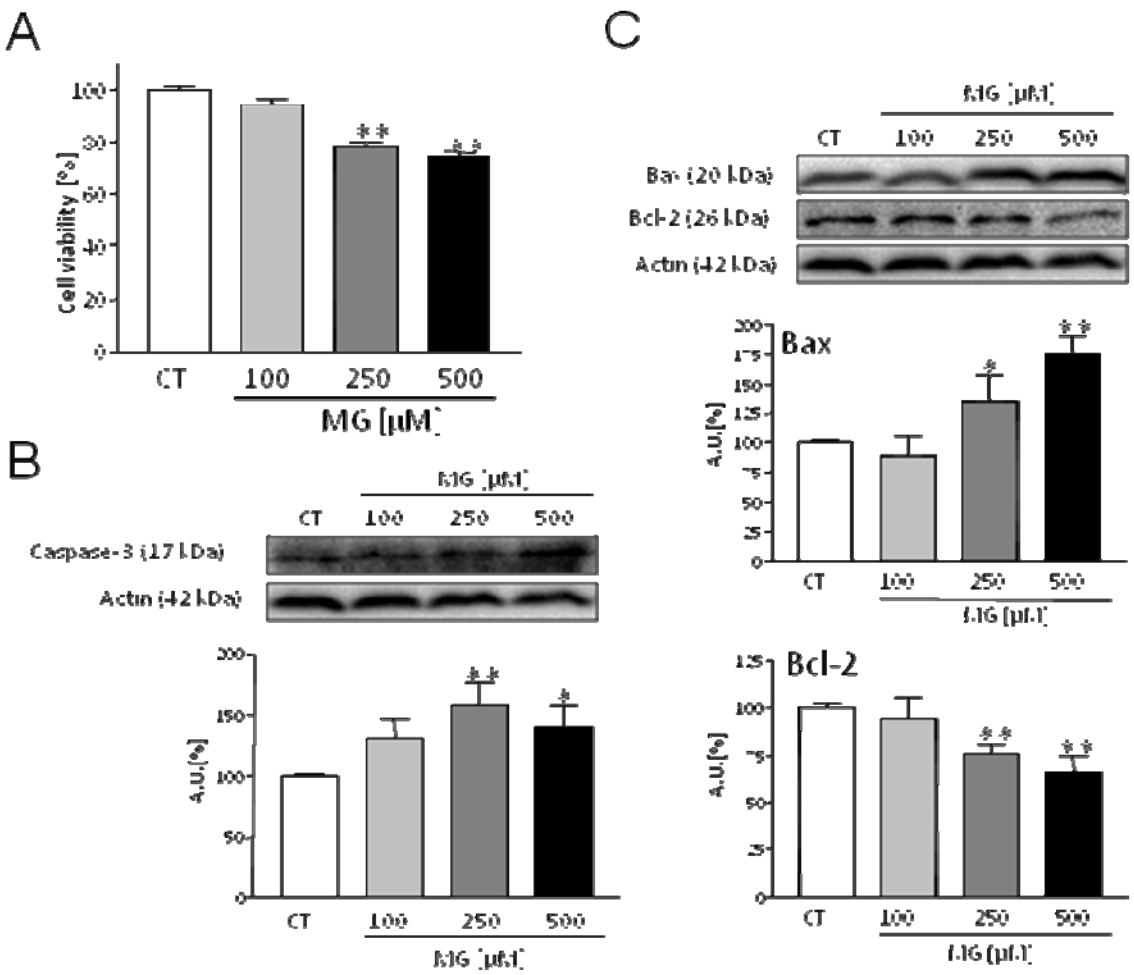
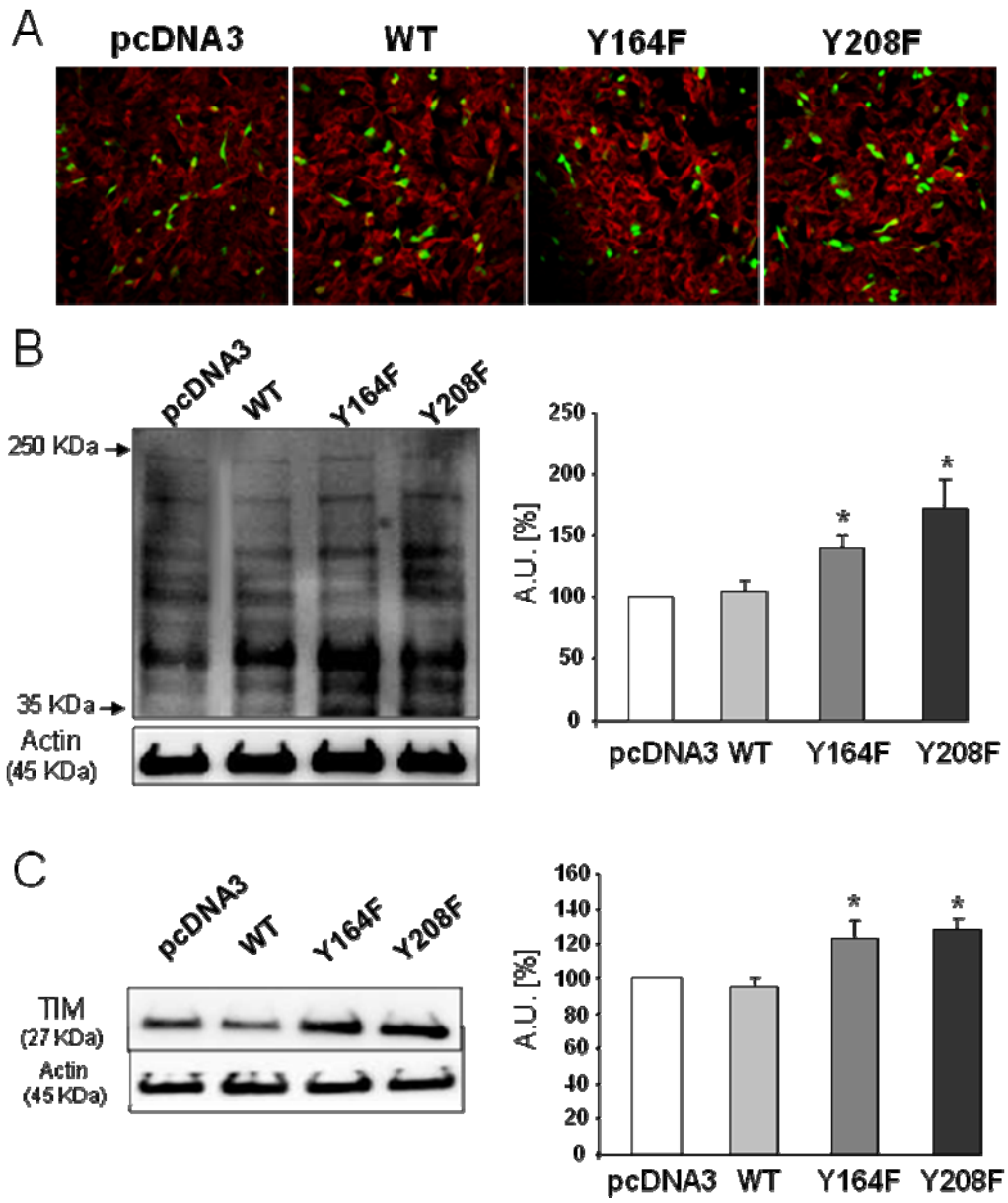

A
Cell viability [%]
CT
100
250
500
MG [μM]
B
MG [μM]
CT
100
250
500
Caspase-3 (17 kDa)
Actin (42 kDa)
A.U.[%]
C
Bax (20 kDa)
Bcl-2 (26 kDa)
Actin (42 kDa)
Bax
Bcl-2

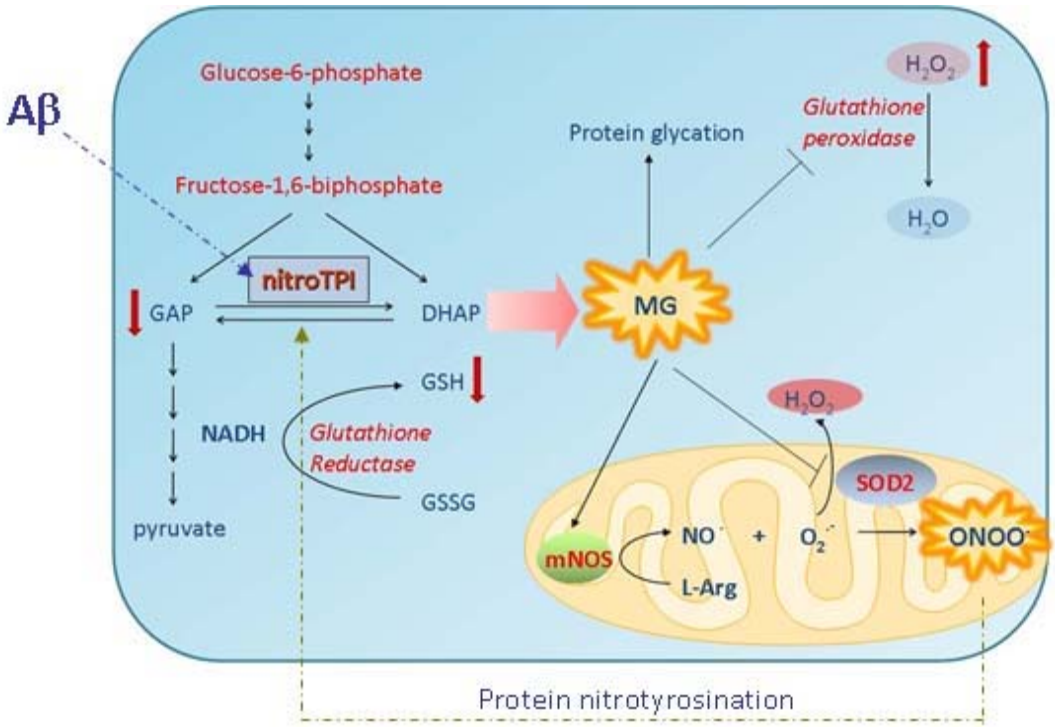
Figure 3


A
pcDNA3
WT
Y164F
Y208F
B
pcDNA3
WT
Y164F
Y208F
250 KDa
35 KDa
Actin
(45 KDa)
A.U. [%]
250
200
150
100
50
0
pcDNA3 WT Y164F Y208F
C
pcDNA3
WT
Y164F
Y208F
TIM
(27 KDa)
Actin
(45 KDa)
A.U. [%]
160
140
120
100
80
60
40
20
0
pcDNA3 WT Y164F Y208F

Supplementary Material

Fig. S1 Intracellular effects of TPI nitrotyrosination. TPI nitrotyrosination compromises its catalytic activity. There is a reduction in the isomerisation of the trioses. This lower rate in the glycolytic pathway produces a decrease in NADH production, avoiding glutathione (GSH) regeneration, a crucial antioxidant defense system of the cell. Moreover, TPI nitrotyrosination induces an increase in methylglyoxal (MG) production. MG binds and inhibits the enzyme glutathione peroxydase, necessary for H_2O_2 elimination (Park et al, 2003). MG has been also proved to increase nitric oxide (NO) synthesis via the stimulation of the mitochondrial NO-synthase (mNOS), and impairing the activity of the detoxifying mitochondrial enzyme Superoxide Dismutase (SOD2) (Wang et al, 2009) affecting to the respiratory mitochondrial chain which triggers superoxide anion ($O_2^{\cdot -}$) production. These two effects combined yield to the formation of the highly reactive peroxynitrite (ONOO-) via the spontaneous reaction between superoxide anion and NO.

Figure S1


Aβ
Glucose-6-phosphate
Fructose-1,6-biphosphate
nitroTPI
GAP
DHAP
MG
Protein glycation
H_2O_2
Glutathione peroxidase
H_2O
GSH
NADH
Glutathione Reductase
GSSG
pyruvate
H_2O_2
SOD2
mNOS
NO˙
+
$O_2^{\cdot-}$
ONOO⁻
L-Arg
Protein nitrotyrosination