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Lack of oestrogen protection in amyloid-mediated endothelial damage due to protein nitrotyrosination

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Amyloid β-peptide (Aβ) cytotoxicity, the hallmark of Alzheimer's disease, implicates oxidative stress in both neurons and vascular cells, particularly endothelial cells. Consequently, antioxidants have shown neuroprotective activities against Aβ-induced cytotoxicity. Among the different antioxidants used in both *in vitro* and *in vivo* studies, 17β-oestradiol (E_2) has garnered the most attention. Oestrogen attenuated $A\beta_{E22Q}$-induced toxicity in neurons but failed to protect endothelial cells. Here we show that E_2-mediated activation of endothelial nitric oxide synthase (eNOS) increases the production of nitric oxide (NO), which, under $A\beta_{E22Q}$-induced oxidative damage, results in the formation of peroxynitrite and increased nitration of tyrosine residues. Inhibition of eNOS prevents nitrotyrosination and permits E_2-mediated protection against $A\beta_{E22Q}$ on endothelial cells. The main nitrotyrosinated proteins in the presence of E_2 and $A\beta_{E22Q}$ were identified by MALDI-TOF mass spectrometry. These proteins are key players in the regulation of energy production, cytoskeletal integrity, protein metabolism and protection against oxidative stress. Our data highlight the potential damaging consequences of E_2 in vascular disorders dealing with oxidative stress conditions, such as cerebral amyloid angiopathy, stroke and ischaemia-reperfusion conditions.

Keywords: Alzheimer's disease; amyloid β-peptide; oestrogen; nitric oxide; nitrotyrosination

Abbreviations: Aβ = amyloid β-peptide; E_2 = 17β-oestradiol; ECs = endothelial cells; eNOS = endothelial nitric oxide synthase; HA-VSMCs = human aortic vascular smooth muscle cells; HUVECs = human umbilical vein endothelial cells; L-NNA = N^G-nitro-L-arginine; NO = nitric oxide; NOS = NO synthase; PTIO = 4,5,5-tetramethylimidazoline-1-oxyl 3-oxide

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Introduction

The vascular pathology associated to Alzheimer's disease resulting in the presence of amyloid β-peptide (Aβ) fibrils in brain vessels is denominated cerebral amyloid angiopathy (CAA) (Ghiso and Frangione, 2001). The hereditary cerebral haemorrhage with amyloidosis of the Dutch type (HCHWA-D) is a familiar kind of CAA. HCHWA-D patients show diffuse amyloid deposits in the brain parenchyma and mature deposits in the brain vasculature, which degenerates, producing haemorrhages (Ghiso and Frangione, 2001). HCHWA-D is produced by a mutation in the Aβ-encoding gene, which causes the replacement of Glu→Gln at position 22 ($A\beta_{E22Q}$), eliciting a more fibrillogenic Aβ than the wild-type (Muñoz *et al.*, 2002). This mutated Aβ has been also demonstrated to be more toxic than the Aβ wild-type (Muñoz *et al.*, 2002).

The cell damage induced by Aβ involves oxidative stress (Butterfield and Bush, 2004). Thus *post mortem* studies showed oxidative markers in lipids, proteins and nucleic acids from Alzheimer's disease patients (Miranda *et al.*, 2000). Moreover, *in vitro* studies have demonstrated the involvement of oxidative stress in Aβ-mediated cytotoxicity in neuronal (Behl, 1997) and vascular cells (Muñoz *et al.*, 2002). Endothelial dysfunction induced by Aβ can be increased by the formation of the powerful nitrating agent peroxynitrite ($ONOO^-$), resulting from the reaction of nitric oxide (NO) with superoxide ($O_2^{\cdot-}$) (Radi, 2004). One of

the consequences of large amounts of peroxynitrite is protein nitrotyrosination, which compromises cellular function and viability (Radi, 2004). Interestingly, massive peroxynitration has been reported in brains from Alzheimer's disease patients (Castegna *et al.*, 2003).

Owing to the involvement of oxidative stress in the pathophysiology of Alzheimer's disease, many therapeutic approaches based on the use of antioxidants have been tested (Miranda *et al.*, 2000). Vitamin E and other antioxidants protect against Aβ-cytotoxicity in neuronal (Butterfield *et al.*, 1999) and vascular (Miranda *et al.*, 2000) cells. The sex hormone 17β-oestradiol (E_2) also protects against Aβ challenge in neurons (Bonnefont *et al.*, 1998), but fails to protect endothelial cells challenged with Aβ or H_2O_2 (Muñoz *et al.*, 2002). E_2 might have many roles in neuroprotection (Behl, 2002), including its neurotrophic (Garcia-Segura *et al.*, 2001) and antioxidant (Moosmann and Behl, 1999) properties. E_2 also presents pleiotropic beneficial effects on the vasculature (Mendelsohn, 2002*b*), particularly favouring vasodilatation by increasing NO bioavailability (Chen *et al.*, 1999). However, this effect might become deleterious under conditions of increased oxidative stress, when NO production by E_2 stimulation could result in excessive peroxynitrite formation.

In the present work we studied the effect of E_2 on $A\beta_{E22Q}$-mediated cytotoxicity in human aortic vascular smooth muscle cells (HA-VSMCs) and human umbilical vein endothelial cells (HUVECs), and primary cultures of mouse cortical neurons and glial cells. We used 10 μM E_2 because the concentration of E_2 acting as an antioxidant *in vitro* is in the micromolar range (Behl, 2002), which is far from the physiological circulating levels but close to the E_2 concentration in microenvironments of the cell membranes where it can be massively inserted. Cell viability, the presence of apoptotic markers and identification of nitrotyrosinated proteins were also assayed in HUVECs challenged with $A\beta_{E22Q}$ and treated with E_2 in the presence of the NO synthase (NOS) inhibitor N^G-nitro-L-arginine (L-NNA) or with the NO scavenger 4,5,5-tetramethylimidazoline-1-oxyl 3-oxide (PTIO), which reacts with NO stoichiometrically and avoids its bioavailability without affecting NOS activity (Akaike *et al.*, 1993).

Material and methods

Materials

Synthetic Aβ peptide corresponding to the human $A\beta_{1–40}$ Dutch variant that contains a glutamic acid to glutamine substitution ($A\beta_{E22Q}$) was purchased from Oncogene (Darmstadt, Germany). $A\beta_{E22Q}$ produces more stable fibrils than Aβ wild-type, but there are no differences in the amyloidogenic properties between the two Aβ types (Muñoz *et al.*, 2002). Amyloid fibrils were obtained and characterized as described previously (Muñoz *et al.*, 2002). $A\beta_{E22Q}$ fibrils were used at a final concentration of 0.125 μM on HUVECs, 0.25 μM on HA-VSMCs, and 1.25 μM on neuronal and glial cells in order to obtain a viability of ~60%. All media and culture products were purchased from Gibco-BRL (Paisley, UK). Experiments were performed with phenol red- and serum-free media. All chemicals were obtained from Sigma (St Louis, MO, USA) unless otherwise indicated.

Cell cultures

HUVECs were grown in M-199 medium supplemented with 10% fetal bovine serum (FBS), 3.2 mM glutamine and antibiotics (100 U/ml penicillin and 10^{-6} μg/ml streptomycin). Mouse lung capillary endothelial cells (1G11 ECs) (kindly provided by Dr. A. Mantovani; Dong *et al.*, 1997) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 20% FBS, 150 μg/ml of endothelial cell growth supplement (Becton-Dickinson, Bedford, MA, USA), 100 μg/ml of heparin, 1% non-essential amino acids and antibiotics. Porcine aortic endothelial cells (PAECs) were grown in DMEM supplemented with 10% FBS and antibiotics. Murine haemangioma endothelial cells (Py-4-1 ECs) were grown in DMEM supplemented with 10% FBS, 2 ng/ml of basic fibroblast growth factor (bFGF) and antibiotics. HA-VSMCs (kindly provided by Dr. S. Richard) were grown in RPMI MCDB 131 medium supplemented with 5% FBS, 5×10^{-7} g/l EGF, 1.5×10^{-6} g/l bFGF, 5 g/l insulin, 2 mM L-glutamine and antibiotics. Mouse cortical neurons were isolated from 18-day-old OS-1 mouse embryos and cultured in DMEM plus B27 (Gibco-BRL) on poly-L-lysine-coated plates. Glial cultures were obtained from 2-day-old mice and cultured in DMEM plus 10% FBS. Cortical neurons were used after 6 days in culture and glial cells after the second passage. Animals were manipulated according to the Council of the European Union (86/6091 EU) and to the ethics committee of the Institut Municipal d'Investigació Mèdica-Universitat Pompeu Fabra (IMIM-UPF).

Brain samples

Brain tissue sections were supplied by the Banc de Teixits Neurològics (Serveis Científico-Tècnics, Hospital Clínic, Universitat de Barcelona). The procedure was approved by the ethics committee of the IMIM-UPF. Brain sections (5 μm) were obtained from the frontal cortex of three control males, seven control females, six males with Alzheimer's disease (stage VI) and six females with Alzheimer's disease (stage VI), none receiving hormone replacement therapy.

Cell viability assay

Cells were seeded in 96-well plates at a density of 8000 cells/100 μl (HUVECs and HA-VSMCs) or 20 000 cells/100 μl (cortical neurons and glial cells) per well. Cells were challenged with $A\beta_{E22Q}$, 10 μM H_2O_2 or PBS. E_2 (0.1 μM or 10 μM) was added 1 h before $A\beta_{E22Q}$ fibrils or H_2O_2. L-NNA (100 μM) or PTIO (10 μM) was added 1 h before E_2 treatment. Trolox (a water-soluble analogue of vitamin E that maintains the OH in the mesomeric ring where the free radical scavenger activity is located and lack of the hydrophobic aliphatic chain of vitamin E; McClain *et al.*, 1995) was used at 100 μM. 17α-oestradiol was used at 1 μM. Oestrogen receptor (ER) antagonists (0.1 μM) ICI 182,780 (a 7α-alkylamide analogue of estradiol with pure anti-oestrogenic activity) or tamoxifen (a non-steroidal triphenylethylene derivative acting as a partial ER antagonist) was added 1 h before E_2. Cells were incubated for 24 h at 37°C and cell viability was measured by the 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) reduction method. Assays were run in triplicate, determined in a Microplate Reader (Model 550; Bio-Rad, Hercules, CA, USA) and data expressed as percentage of control.

Apoptosis assay

HUVECs (4×10^4) were seeded on 1% poly-L-lysine-coated coverslips and treated for 6 h at 37°C with $A\beta_{E22Q}$, E_2 and L-NNA or PTIO. Coverslips were processed with the DeadEnd™ Colorimetric TUNEL System (Promega, Madison, WI, USA). Representative digital images were taken with a Leica DMRB and analysed with CCD Leica DC300F (Heidelberg, Germany).

Akt phosphorylation

HUVECs (1.5×10^6) were seeded on 90-mm plates and treated for 24 h at 37°C with $A\beta_{E22Q}$ or E_2. Cells were lysed and 75 μg protein per sample was run in 10% SDS–PAGE. Blotted nitrocellulose membranes were incubated with 1 : 500 mouse anti-phospho-Akt (Ser473) monoclonal antibody (Ab) (Cell Signaling, Beverly, MA, USA) overnight at 4°C or rabbit anti-β-actin monoclonal Ab for 1 h at room temperature as loading control. It was followed by incubation with 1 : 5000 sheep anti-mouse peroxidase-conjugated polyclonal Ab or donkey anti-rabbit peroxidase-conjugated polyclonal Ab (Amersham Bioscience, Barcelona, Spain) for 1 h at room temperature. Bands were visualized using the enhancer chemiluminescence substrate Super Signal (Pierce, Rockford, IL, USA) and Amersham Bioscience Hyperfilm ECL kit.

Nitrotyrosine immunoreactivity on endothelial cells

HUVECs, PAECs, 1G11 ECs and Py-4-1 ECs (4×10^4) were seeded on 1% poly-L-lysine coated coverslips and treated for 24 h at 37°C with $A\beta_{E22Q}$, E_2, sodium nitroprussiate (SNP) and L-NNA or PTIO. Cells were fixed and incubated for 2 h at room temperature with 1 : 500 rabbit anti-nitrotyrosine polyclonal Ab (Molecular Probes, Leiden, The Netherlands) followed by incubation with 1 : 500 Alexa Fluor 488 goat anti-rabbit polyclonal Ab for 1 h at room temperature. Digital images were taken with a Leica TCS SP confocal microscope and analysed with Leica confocal software (Heidelberg, Germany).

Brain sample staining

Sections were treated with alkaline solution followed by Congo Red staining. The following sections were treated with 4% H_2O_2 and incubated with 1 : 500 rabbit anti-nitrotyrosine polyclonal Ab for 2 h at room temperature followed by incubation with 1 : 5000 biotinilated goat anti-rabbit polyclonal Ab (DAKO, Glostrup, Denmark) for 1 h at room temperature. Slides were incubated with streptavidin–horseradish peroxidase (Zymed Laboratories, San Francisco, CA, USA) and treated with Peroxidase Substrate Kit DAB (Vector, Burlingame, CA, USA). Samples were counterstained with haematoxylin, dehydrated and fixed with Eukitt (O. Kindler, GmbH., Fribourg, Switzerland). Representative digital images were taken and analysed as described above.

NO assay

HUVECs (1.5×10^6) seeded on 90-mm plates were treated with $A\beta_{E22Q}$, E_2 and L-NNA or PTIO for 24 h. Cells were lysed and protein concentration determined by the Bio-Rad protein assay. NO was measured (40 μl samples in triplicate) using a nitrate/nitrite colorimetric assay kit (Cayman, Ann Arbor, MI, USA). NO production was calculated to the amount of protein.

Identification of nitrotyrosinated proteins

HUVECs (1.5×10^6) were seeded on 90-mm plates and treated for 24 h at 37°C with $A\beta_{E22Q}$, E_2 and L-NNA or PTIO. Cells were lysed in 100 μl buffer (7 M urea, 2 M thiourea, 4% CHAPS, 2% IPG buffer 3–10 NL, 1% DTT and protease inhibitors), sonicated, acetone precipitated and subsequently centrifuged.

2D gel electrophoresis

Protein (200 μg) was dissolved up to 125 μl buffer (7 M urea, 2 M thiourea, 4% CHAPS, 0.5% IPG buffer 3–10 NL and 1.2 ml Destreak reagent). Isolectric focusing was carried out at 20°C. The rehydration step was carried out for 12 h at 50 V, and was followed by 1 h at 200 V, 30 min at 500 V, 30 min at 1000 V, a 40 min gradient until 5000 V, and 4 h at 5000 V. Gel strips were equilibrated in DTT and iodoacetamide-based buffers and loaded onto 12% SDS–PAGE. Duplicate gels were run for each sample, one for western blot and another for protein identification.

Western blot

Nitrocellulose membranes were incubated for 2 h at room temperature with 1 : 500 rabbit anti-nitrotyrosine polyclonal Ab and for 1 h at room temperature with 1 : 5000 donkey anti-rabbit peroxidase-conjugated polyclonal Ab (Amersham Bioscience). Bands were visualized as described above. The protein identification was performed in gels stained with Coomassie R-350. Bands matching those shown to contain nitrotyrosine by western blot in the duplicate set (see above) were unstained by sequential hydration/dehydration steps with 0.1 M NH_4HCO_3 (pH 8) and acetonitrile, respectively. Gel plugs were dried in a Speed-Vac for 5 min. Each spot was treated with 100 ng sequencing-grade trypsin in 50 mM NH_4HCO_3 and incubated for 30 min at 4°C and then overnight at 37°C. Digested samples (10 μl) were desalted with a Poros R2 column (ABI). Peptide mass fingerprints were obtained in a Voyager DE-STR MALDI-TOF mass spectrometer (Applied Biosystems, Foster City, CA, USA) and searched against the NCBI and Swiss-Prot protein databases using the MASCOT search engine. Peptide mass fingerprinting used the assumption that peptides were monoisotopic, oxidized at Met residues and carbamidomethylated at Cys residues. A mass tolerance of 50 ppm was the window error allowed for matching the peptide mass values.

Statistical analysis

Data are expressed as the mean ± SEM of the values from the number of experiments as indicated in the corresponding figures. Data were evaluated statistically using Student's *t*-test or one-way ANOVA, followed by Bonferroni's *post hoc* analysis. The level of significance was $P < 0.05$.

Results

E_2-mediated protection against $A\beta_{E22Q}$ cytotoxicity in endothelial cells requires low NO bioavailability

$A\beta_{E22Q}$ fibrils from the Dutch variant induced marked cytotoxicity in vascular and neuronal cells (Fig. 1). E_2 reverted the cytotoxic effect of $A\beta_{E22Q}$ on cortical neurons and HA-VSMCs (Fig. 1A). However, it failed to revert $A\beta_{E22Q}$ toxicity on HUVECs (Fig. 1B). One possible explanation for the differential protective effect of E_2 is related to NO bioavailability.

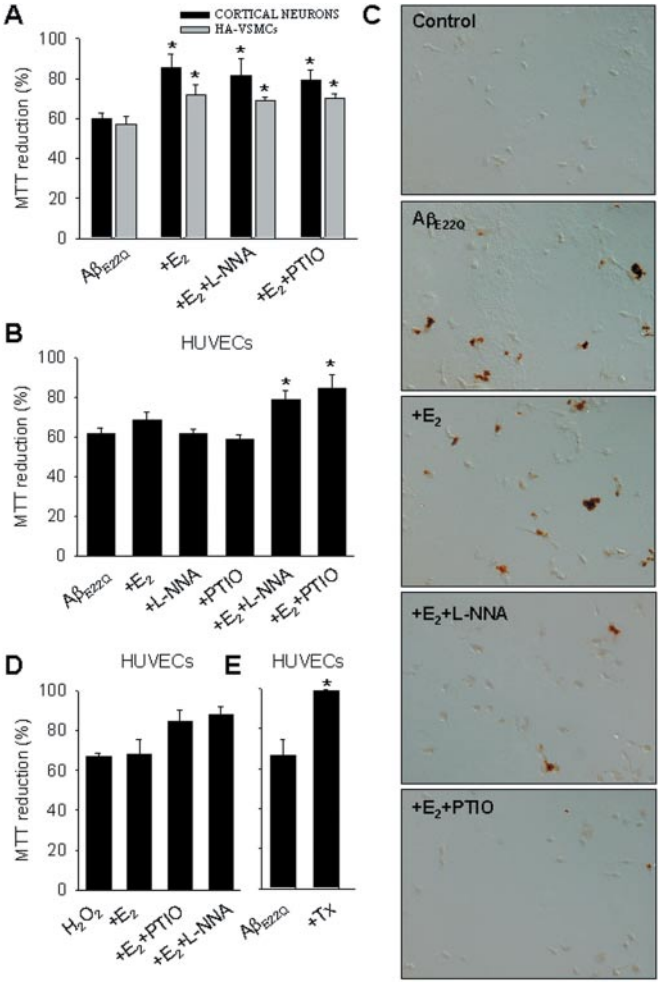

Fig. 1 Effect of E_2 in Aβ-mediated cytotoxicity. E_2 (10 μM) protected HA-VSMCs and cortical neurons (**A**) against 0.25 μM (for HA-VSMCs) and 1.25 μM (for neurons) $A\beta_{E22Q}$ independently of 100 μM L-NNA and 10 μM PTIO. Data are mean ± SEM values of three to seven experiments. HUVECs were challenged with 0.125 μM $A\beta_{E22Q}$ fibrils (**B**) and protection with 10 μM E_2 was obtained only when NO inhibitors were present. Data are mean ± SEM values of seven to nine experiments. HUVECs were treated and stained by TUNEL method detecting apoptotic cells (**C**). Pictures were taken from representative experiments (n = 3) performed in duplicate. HUVECs were challenged with 10 μM H_2O_2 (**D**) in a representative experiment performed in triplicate and protection by E_2 was obtained solely in the presence of NO inhibitors. 100 μM Trolox protected $A\beta_{E22Q}$ challenge on HUVECs (**E**). *$P < 0.05$.

Under physiological conditions NO is a powerful vasodilator but under oxidative conditions it becomes harmful to the cells due to peroxynitrite formation. E_2 is a well-known activator of endothelial nitric oxide synthase (eNOS) (Chen *et al.*, 1999). Therefore, we evaluated the protective effect of E_2 against $A\beta_{E22Q}$ in the presence of the NOS inhibitor L-NNA and the NO scavenger PTIO (Fig. 1B). Under both conditions, E_2 significantly reduced the $A\beta_{E22Q}$-mediated cytotoxicity in HUVECs measured by MTT assay ($P < 0.05$). On the other hand, the presence of L-NNA or PTIO did not modify the cell viability of HA-VSMCs or cortical neurons (Fig. 1A). Interestingly, experiments carried out in mouse glial cells, which express the inducible isoform of NOS (iNOS), which is not regulated by E_2 (Fulton *et al.*, 1999), showed that E_2 increased cell viability (75 ± 9%) in response to $A\beta_{E22Q}$ (57 ± 7%), and this protection was further increased (86 ± 13%) in the presence of 100 μM aminoguanidine, a specific inhibitor of iNOS.

$A\beta_{E22Q}$ toxicity, like oxidative stress in general, has been associated with the induction of apoptotic cell death (Muñoz *et al.*, 2002). Accordingly, the presence of apoptotic endothelial cells in response to $A\beta_{E22Q}$ and the different treatments was tested using the TUNEL assay (Fig. 1C). Under control conditions no apoptotic HUVECs were observed in a representative optical field. Treatment with $A\beta_{E22Q}$ induced the appearance of numerous apoptotic cells that was not prevented by E_2, but was reverted by coincubation with E_2 plus L-NNA or PTIO. Altogether, the results obtained using MTT and TUNEL assays indicate that the protective effect of E_2 against $A\beta_{E22Q}$ is only achieved by inhibiting the eNOS or reducing the availability of NO with a NO scavenger. The same pattern of response was obtained when HUVECs were incubated with 10 μM H_2O_2, a pro-oxidant stimulus (Fig. 1D). The most significant protection against $A\beta_{E22Q}$ toxicity was provided by Trolox, a powerful antioxidant (Fig. 1E).

ER-independent protection by E_2

Akt phosphorylation was observed in HUVECs exposed to E_2 (Fig. 2E). Slight activation of Akt was also observed in cells treated with $A\beta_{E22Q}$. Since the PI3K/Akt pathway has been involved in the maintenance of cell survival (Franke *et al.*, 2003), this effect could be related to the triggering of protective mechanisms.

The role of ERα in the effect of E_2 in $A\beta_{E22Q}$-mediated cytotoxicity was assayed in HUVECs exposed to $A\beta_{E22Q}$ or $A\beta_{E22Q}$ plus E_2 in the presence of the ERα antagonists ICI 182,780 and tamoxifen (Fig. 2A). ER inhibition provides protection by E_2 against the $A\beta_{E22Q}$ challenge in HUVECs ($P < 0.05$). Under these conditions, eNOS cannot be activated and the increased NO supply is prevented. On the other hand, the use of ER inhibitors did not modify the protective effect of E_2 on HA-VSMCs (Fig. 2B). Since E_2 activates eNOS at physiological concentrations, E_2 was also assayed at 0.1 μM (Fig. 2C), but protection was not obtained in the presence of L-NNA or PTIO. At this low concentration E_2 lacks its antioxidant ability. Accordingly, HUVECs challenged with $A\beta_{E22Q}$ in the presence of 1 μM 17α-oestradiol (Fig. 2D), which does not bind ER but maintains the antioxidant properties, were protected. Altogether, the data shown suggest that E_2 protection is independent of its binding to the ER.

$A\beta_{E22Q}$ and $A\beta_{E22Q}$ plus E_2 induce nitrotyrosination of protein residues that can be reverted by NO inhibitors

Excessive NO production induced by E_2-dependent activation of eNOS reduces the protective effect of E_2 in HUVECs

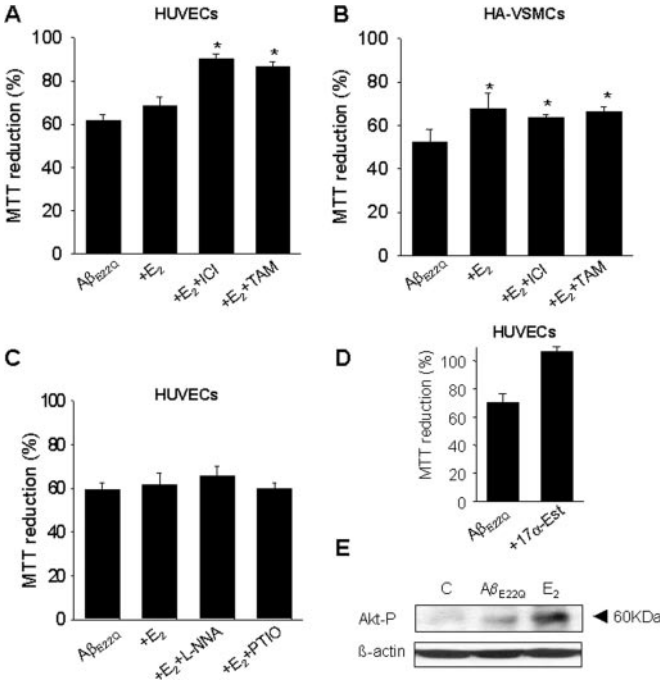

Fig. 2 The role of ER on E_2 effect. HUVECs (**A**) and HA-VSMCs (**B**) were challenged with $A\beta_{E22Q}$ fibrils and treated with E_2 in the presence of 0.1 μM ICI 182,780 and 0.1 μM tamoxifen. HUVECs were also challenged with $A\beta_{E22Q}$ fibrils and 0.1 μM E_2 in the presence of 100 μM L-NNA and 10 μM PTIO (**C**). Data are mean ± SEM values of four to nine experiments. *$P < 0.05$. A representative study on HUVECs was carried out with $A\beta_{E22Q}$ fibrils and 1 μM 17α-oestradiol (**D**). Western blot analysis of phospho-Akt (Ser 473) expression was performed on HUVECs after 24 h with $A\beta_{E22Q}$ fibrils and E_2 (**E**).

challenged with $A\beta_{E22Q}$. This finding could be related to the production of peroxynitrites in a pro-oxidant environment. Therefore, we studied protein nitrotyrosination in HUVECs (Fig. 3A, left panels). We observed that in the absence of oxidative challenge HUVECs presented a low level of nitrotyrosination, determined by confocal immunofluorescence with an anti-nitrotyrosine Ab. $A\beta_{E22Q}$ induced a significant increase in nitrotyrosination that further increased in the presence of E_2 and was reverted by L-NNA or PTIO. Identical results were obtained with other ECs: 1G11 ECs (Fig. 3B), PAECs and Py-4-1 ECs (data not shown). Moreover, $A\beta_{E22Q}$ challenged with a NO donor (sodium nitroprussiate) mimics the result obtained with $A\beta_{E22Q}$ plus E_2 treatment on HUVECs (Fig. 3D). The nitrotyrosination levels were transformed into a pseudocolour scale (Fig. 3A, right panels) and represented quantitatively as fluorescence arbitrary units (Fig. 3C). Figure 3C also shows the NO levels as percentage relative to control conditions. $A\beta_{E22Q}$ challenge, in addition to increasing nitrotyrosination of HUVECs, reduced the NO level, most likely owing to the formation of peroxynitrite following the reaction of NO with the superoxide anion generated by the presence of $A\beta_{E22Q}$. The highest levels of nitrotyrosination were observed in HUVECs exposed to $A\beta_{E22Q}$ plus E_2, in agreement with the highest NO production. Both nitrotyrosine fluorescence and NO levels were reduced in cells exposed to L-NNA or PTIO in the presence of $A\beta_{E22Q}$ plus E_2. Nitrotyrosine formation has been described in Alzheimer's disease brains (Castegna *et al.*, 2003). Therefore, we also evaluated nitrotyrosination in the frontal cortex vessels from Alzheimer's disease patients (Fig. 3E). Immunohistochemical studies revealed the absence of either Aβ vascular deposits or nitrotyrosination reactivity in sequential sections from controls without Alzheimer's disease (Fig. 3E, top panels). In constrast, in brain sections from Alzheimer's disease patients, Aβ vascular deposits correlating with protein nitrotyrosination were observed (Fig. 3E, bottom panels), suggesting that Aβ-mediated vascular damage is associated to protein nitrotyrosination. No differences in the presence of nitrotyrosination associated to amyloid deposits were observed attending to the gender.

Identification of nitrotyrosinated proteins in HUVECs

In order to investigate the main target proteins for nitrotyrosination, we carried out a comparative 2D elecrophoretic analysis by western blot (Fig. 4). While no nitrotyrosine immunoreactivity was detected in control HUVECs (Fig. 4A), clear nitrotyrosination was present in HUVECs treated with $A\beta_{E22Q}$ (Fig. 4B), which was even more prominent in cells treated with $A\beta_{E22Q}$ and E_2 (Fig. 4C). The addition of L-NNA (Fig. 4D) or PTIO (Fig. 4E) significantly reduced the nitrotyrosine immunoreactivity. The nitrotyrosinated proteins identified in the presence of $A\beta_{E22Q}$ or $A\beta_{E22Q}$ plus E_2 are listed in Table 1.

Discussion

Oestrogens have been widely proposed as neuroprotective agents in a variety of *in vivo* and *in vitro* models (Behl, 2002; Mendelsohn, 2002*a*). Moreover, clinical trials have associated E_2 with the retardation of the onset and progression of Alzheimer's disease (Tang *et al.*, 1996; Kawas *et al.*, 1997), although other studies offered a less optimistic scenario (Grodstein *et al.*, 2000; Rapp *et al.*, 2003). The putative neuroprotective effects of E_2 in Alzheimer's disease involve decreased Aβ production, enhanced synthesis of choline-acetyltransferase, promotion of neuronal growth (Garcia-Segura *et al.*, 2001), activation of potassium channels leading to vasodilatation (Valverde *et al.*, 1999) and antioxidant properties (Moosmann and Behl, 1999). The antioxidant effect of E_2 is independent of its interaction with ERs or other oestrogen-binding sites and is related to the phenolic OH group (Moosmann and Behl, 1999), a chemical structure also present in α-tocopherol. Antioxidants inhibit membrane lipid peroxidation elicited by free radicals (Butterfield *et al.*, 1999) as well as the intracellular damage triggered by Aβ (Behl *et al.*, 1994).

Our study shows a cell type-dependent protective effect of oestrogen against $A\beta_{E22Q}$-mediated cytotoxicity. Oestrogen is

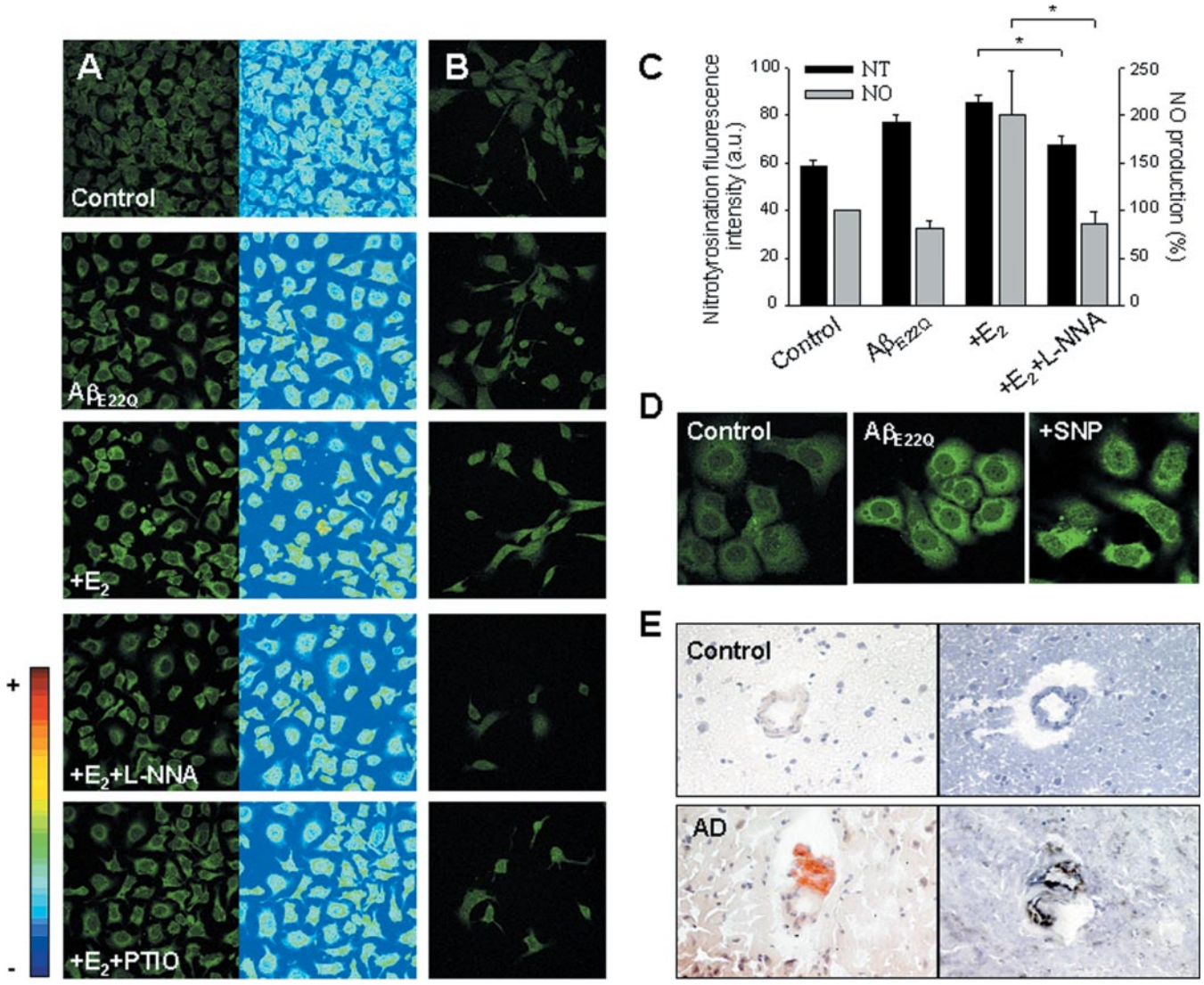

Fig. 3 Production of NO and nitrotyrosination in HUVECs. Protein nitrotyrosination on HUVECs is indicated by green staining and nitrotyrosination intensity is shown using a pseudocolour scale (**A**). Protein nitrotryrosination on 1G11 ECs is indicated by green staining (**B**). Fluorescence intensity from HUVECs is represented as arbitrary units (NT; black bars) and NO production from HUVECs (NO; grey bars) is expressed as percentage of control cells (**C**). Data are mean ± SEM values of seven to 10 separate experiments. *$P < 0.05$. Nitrotyrosination was also studied by using a NO donor (SNP) plus $A\beta_{E22Q}$ fibrils on HUVECs (**D**). Colocalization of vascular amyloid deposits with nitrotyrosination (**E**) was performed in control and Alzheimer's disease patient brains. Representative pictures from female brain samples are shown.

able to protect cortical neurons, glial cells and smooth muscle cells against $A\beta_{E22Q}$, but fails to protect ECs. Oestrogen uses both genomic and alternative (non-genomic) mechanisms of action that might implicate the known ER (ERα and ERβ) (Nadal *et al.*, 2001) or be ER-independent (e.g. its antioxidant effect) (Behl, 2002). In the endothelium, eNOS produces NO by the conversion of L-arginine to L-citrulline (Radi, 2004). eNOS is activated by E_2 via the ERα and PI3K/Akt pathway within caveoli signalling microdomains (Mendelsohn, 2002*a*). HUVECs express ERα that colocalizes with caveolin-1 (data not shown). The lack of protection on endothelial cells is related to the E_2-dependent activation of eNOS and the production of NO as inhibition of ER or eNOS enables the protective effect of E_2. These results suggest that the protective role of E_2 is independent of its binding to the ER but related to its antioxidant properties. The fact that none of the cells treated with E_2 conferred protection against $A\beta_{E22Q}$ at nanomolar concentrations is also suggestive of a mechanism of action different from its interaction with ERs. NO reacts with the superoxide anion producing highly reactive peroxynitrite (Radi, 2004), which causes protein nitrotyrosination, a marker of cell damage reported in neurons and glial cells from Alzheimer's disease brains (Castegna *et al.*, 2003). We have found that vascular amyloid deposits correlate with nitrotyrosination in brain vessels from Alzheimer's disease patients. These findings are in agreement with previous studies describing endothelial cell degeneration in CAA (Miyakawa *et al.*, 1997) and dysfunction of the

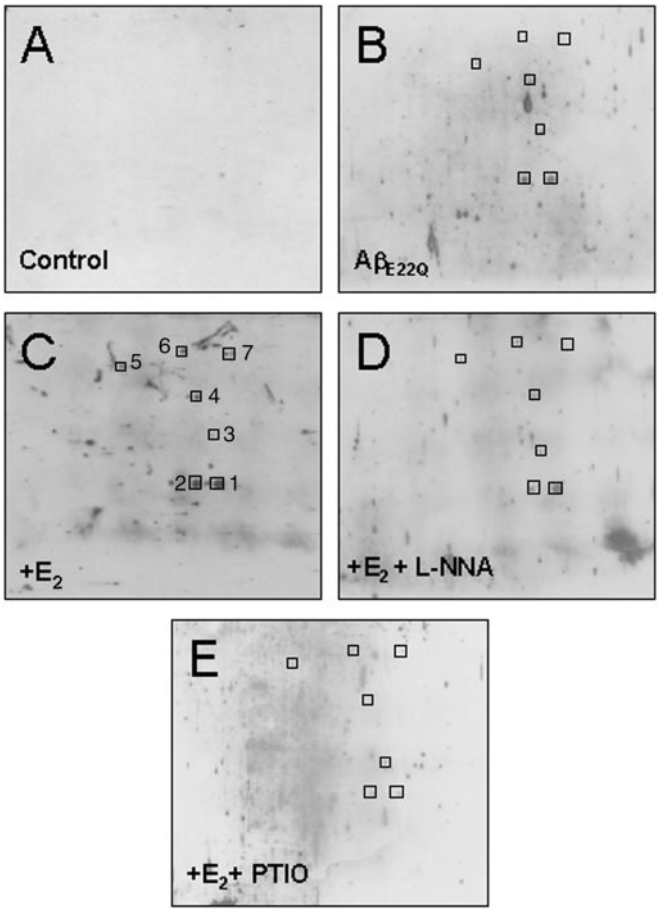

Fig. 4 Identification of nitrotyrosinated proteins. Western blots show nitrotyrosinated proteins from HUVECs (**A**) challenged with $A\beta_{E22Q}$ (**B**) and 10 μM E_2 (**C**) in the presence of 100 μM L-NNA (**D**) and 10 μM PTIO (**E**). The proteins identified to be nitrotyrosinated were triose phosphate isomerase (1), peroxiredoxin 6 (2), 26S proteasome (3), T-complex protein (4), MTHSP75 (5), Metavinculin (6) and eukaryotic translation elongation factor 2 (7).

blood–brain barrier (Wisniewski *et al.*, 2000). No difference in the level of nitrotyrosination associated to amyloid plaques was observed between samples from both genders. This fact may be explained based on the low levels of circulating oestrogens present in postmenopausal women (Orshal and Khahil, 2004).

Aβ fibrils act as a source of superoxide anion (Butterfield and Bush, 2004), which can react with the basal levels of NO, owing to the high affinity of NO for the superoxide anion (Huie and Padmaja, 1993), triggering nitrotyrosination. We have observed protein nitrotyrosination in HUVECs exposed to $A\beta_{E22Q}$ alone. Higher levels of nitrotyrosination were observed in HUVECs exposed to $A\beta_{E22Q}$ and E_2, an effect reversed by PTIO and L-NNA. However, no increase in cell viability was seen in the presence of $A\beta_{E22Q}$ and PTIO or L-NNA, suggesting that the main source of cell damage is provided by the Aβ-induced oxidative stress, rather than nitrotyronization. Alternatively, it might be necessary to reach a nitrotyrosination threshold in order to produce cell death, as suggested previously (Paris *et al.*, 1998).

In this study, we have identified several proteins that are nitrotyrosinated under the conditions we have tested (Table 1). They are functionally related to the regulation of energy production, cytoskeletal integrity, protein metabolism and protection against oxidative stress. The functions of these proteins should be inhibited since nitrotyrosination has been mainly associated with the loss of function and subsequent labelling for degradation via the proteasome (Grune *et al.*, 1998).

One of the most striking proteins to be nitrotyrosinated, TIM, is involved in the interconversion of dihydroxyacetone phosphate (DHAP) and glyceraldehyde-3-phosphate (GA3P) in the glycolytic pathway. If TIM function is altered, DHAP will be accumulated in the cell. Interestingly, inherited TIM deficiency leads to abnormal accumulation of DHAP and chronic neurodegeneration and has been also associated to degeneration of vascular endothelial cells (Ahmed *et al.*, 2003). It has also been proposed that a defective TIM could form pathological aggregates with microtubules (Orosz *et al.*, 2000).

Mitochondrial HSP75 plays an important role in preserving the integrity of mitochondria. This chaperone assists the folding of imported proteins to this organelle, as well as those proteins altered by oxidative stress. The inactivation of this enzyme is a key step in producing the mitochondrial impairment, failure of energetic metabolism and cerebral hypoperfusion (Aliev *et al.*, 2003).

The non-selenium GluPx (or 1-cys peroxiredoxin) is a cytosolic bifunctional enzyme with peroxidase and PLA_2-like activities (Chen *et al.*, 2000). It hydrolyses phospholipid hydroperoxides to free fatty acids hydroperoxides, playing an important role in preserving the membrane function and integrity (Fisher *et al.*, 1999). Its nitrotyrosination could produce an increase in the oxidative injury.

TCP-1 is a cytosolic chaperone that assists in the folding of tubulin, actin and vinculin. The abnormal folding of cytoskeletal proteins in endothelial cells might determine alterations in cell adhesion, loss of the blood–brain barrier selectivity and endothelial apoptosis (Li *et al.*, 1999).

Eukaryotic translation elongation factor 2 (ef-2) is responsible for the elongation phase during protein synthesis. It has been reported that oxidative stress reduces protein synthesis (Patel *et al.*, 2002), thereby nitrotyrosination of ef-2 might result in reduced protein synthesis.

Finally, 26S proteasome is one of the main degradation systems inside the cell (Goldberg, 2003). The nitrotyrosination of 26S proteasome could alter the degradation of proteins in a critical situation as oxidative stress.

In conclusion, our study shows that the beneficial effect of E_2 against Aβ-mediated cell damage in endothelial cells is ER-independent, while its endothelial harmful effect is through its interaction with ER, via NO production and protein nitrotyrosination. Although there is not increased cell death in the presence of $A\beta_{E22Q}$ and E_2 compared with $A\beta_{E22Q}$ alone, there

Table 1 Identified nitrotyrosinated proteins

Protein no.		M_r (Da)	pI	SC (%)	Function
1	Triose phosphate isomerase (chain A)	26 807 (T) 28 000 (O)	6.51 (T) 6.40 (O)	69	Glycolysis (Cyt)
2	Peroxiredoxin 6, non-glutathione peroxidase	25 133 (T) 28 000 (O)	6.00 (T) 6.20 (O)	54	Detoxification of free radicals (Cyt and Lys)
3	26S Proteasome	45 768 (T) 40 000 (O)	7.11 (T) 6.60 (O)	44	Protein degradation (Cyt and Nuc)
4	TCPB (T-complex protein 1, β-subunit)	57 794 (T) 54 000 (O)	6.01 (T) 6.00 (O)	63	Chaperone of actin and tubulin (Cyt)
5	MTHSP75	73 920 (T) 70 000 (O)	5.87 (T) 5.70 (O)	17	Chaperone of mitochondrial proteins (Mit)
6	Metavinculin	124 161 (T) 116 000 (O)	5.51 (T) 5.90 (O)	25	Cytoskeleton (Submemb)
7	Eukaryotic translation elongation factor 2	96 246 (T) 96 000 (O)	6.41 (T) 6.60 (O)	21	Protein translation (Cyt)

T = theoretical; O = observed; SC = sequence coverage; Cyt = cytoplasmic; Lys = lysosomal; Nuc = nuclear; Mit = mitochondrial; Submemb = submembranal.

is a significant increase of nitrotyrosination in enzymes involved in glucose metabolism, energetic balance, repairing systems, protein degradation and cytoskeleton, which most likely compromise cell functions.

Oestrogen effects are complex, with many preliminary studies praising its neuro- and vascular-protective effects (Behl, 2002; Mendelsohn, 2002*a*), whereas clinical trial have yielded disappointing results (Grodstein *et al.*, 2000; Viscoli *et al.*, 2001; Hippisley-Cox *et al.*, 2003; Rapp *et al.*, 2003). Our data suggest possible damaging effects of E_2 in vascular disorders dealing with oxidative stress conditions, such as cerebral amyloid angiopathy (Muñoz *et al.*, 2002), stroke and ischaemia-reperfusion conditions (Gilgun-Sherki *et al.*, 2002), where an overproduction of NO can be harmful (Hobbs *et al.*, 1999). They might also cast light on the mechanisms that will explain recently reported worsening of the injury caused by recurrent cerebral ischaemia in women undergoing hormone replacement therapy (Viscoli *et al.*, 2001; Rossouw *et al.*, 2002).

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