

Amyloid-Beta induces different expression pattern of tissue transglutaminase and its isoforms on Olfactory Ensheathing Cells: modulatory effect of Indicaxanthin

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Abstract

Alzheimer Disease (AD) is characterized by protein aggregates in the brain, including amyloid-beta ($A\beta$), a substrate for tissue transglutaminase (TG2). We assessed the effect of full native peptide of $A\beta$ (1–42), the fragments (25–35 and 35–25) on TG2 expression and its isoforms (Long and Short) on mouse Olfactory Ensheathing Cells (OECs). The levels of cytoskeletal proteins, Vimentin and Glial Fibrillary Acid Protein, were also studied. The effect of the pre-treatment with Indicaxanthin on cell viability, total Reactive Oxygen Species, superoxide anion and apoptotic pathway activation was assessed. Since Nestin is co-expressed in pluripotent stem cells with cyclin D_1 , their levels were also evaluated. Our findings highlight that $A\beta$ (1–42) and its fragments induced an increase of TG2 levels and the different expression pattern of its isoforms on OECs. Indicaxanthin pre-treatment was able to reduce TG2 over-expression upregulated by $A\beta$ exposure of cells differently modulating its isoforms. In addition, it prevented total Reactive Oxygen Species and superoxide anion production, it reduced Glial Fibrillary Acid Protein and Vimentin levels and inhibited apoptotic pathway activation. It also led to an increase of Nestin and cyclin D_1 expression, stimulating stem cells renewal through the reparative activity played by TG2. Our results suggest that $A\beta$ in OECs, both in the absence and in the presence of Indicaxanthin, might differently induce the transition of TG2 between “closed” and “open” conformation, providing a new mechanism involved in the signal pathways activated by the protein in $A\beta$ injury important for neural regeneration of AD.

Introduction

Alzheimer Disease (AD) is characterized by intracellular and extracellular protein aggregates in the brain, including microtubule-associated protein tau and cleavage products of the amyloid precursor protein, amyloid-beta ($A\beta$). The accumulation of $A\beta$ is responsible of oxidative stress, inflammation and neurotoxicity, that lead to apoptosis and the deterioration of the neurotransmission system observed in AD [1]. $A\beta$ is also a substrate for tissue transglutaminase (TG2), an ubiquitarian calcium-dependent protein that catalyses cross-linking reactions, inducing $A\beta$ oligomerization and aggregation, which are typical signs of AD [2]. It also has disulfide isomerase [3], kinase and GTPase activities [4]. TG2 is mainly localized in the cytosol (73%), partially in the plasma membrane (20%), nucleus (7%), and the extracellular matrix [5–7]. The functions of TG2 depend on its intracellular localization. When it is localized in the cytosol, TG2 controls apoptotic processes in a stimuli-dependent manner, through its transamidating activity that is necessary for pro-apoptotic effects [6]. Instead, when TG2 is localized into the nuclear compartment, it phosphorylates different proteins, including retinoblastoma and p53, that are known to be substrates for TG2 kinase activity [8]. Furthermore, TG2 is present in two isoforms: short TG2 (TG2-S) and long TG2 (TG2-L), having different cellular localization that mediate opposite cellular functions [9–11]. In particular, TG2-S, localized in the cytosol and mitochondria, increases during apoptosis, is responsible of the aggregate formation and involved in AD [9–11]. In contrast, TG2-L, localized in the nucleus, exerts a protective effect against cellular injury and apoptosis due to its transamidating activity [9–11].

Several findings reported that in AD patients an early sign of neurodegeneration is represented by a reduced function of olfactory performance [12]. In particular, a peculiar olfactory glial cell is represented by Olfactory Ensheathing Cells (OECs). This cellular type, showing a bipolar or multipolar morphology, surrounds the olfactory nerves, is able to secrete different growth factors, neurotrophins, adhesion molecules and numerous markers, which promote neuron survival and axonal growth, supporting also injured Central Nervous System [13–16]. OECs are able to stimulate angiogenesis and remyelination, therefore they play an important role in transplants in spinal cord injury [17]. In addition, OECs exhibit stem cell properties, expressing Nestin, a marker of precursor neural stem cells [17]. In previous researches we demonstrated that TG2 was overexpressed in OECs exposed to full native peptide A β (1–42) and A β (25–35) fragment and that some growth factors were able to down-regulate the expression levels of the protein [18].

In the recent years, growing attention rose on neuro-nutraceuticals, such as Indicaxanthin, a phytochemical produced by cactus pear fruit from *Opuntia ficus-indica*, L. Mill. [19, 20]. Indicaxanthin possesses significant anti-proliferative, antitumor and anti-inflammatory effects both *in vivo* and *in vitro* [21, 22]. In addition, it modulates reactive oxygen species (ROS) production, prevents mitochondrial damage, regulates cell redox balance and calcium homeostasis in a number of experimental *in vitro* models [23]. Interestingly enough, and in contrast with the majority of phytochemicals, Indicaxanthin is able to cross the blood brain barrier and to modulate the bioelectric neuronal activity in the hippocampus [24].

Herein, we assessed TG2, TG2-S and TG2-L expression levels in OECs exposed to A β (1–42) or A β (25–35) or reverse-sequence fragment A β (35–25) [25] and the effect of Indicaxanthin. The expression levels of some cytoskeletal proteins, such as Vimentin, marker of gliosis and also substrate of TG2, Glial Fibrillary Acid Protein (GFAP), glial marker of growth, maturation and differentiation, were evaluated. Since Nestin, marker of neural precursors, is co-expressed in pluripotent stem cells with cyclin D₁ [26], marker of cellular proliferation, the effect of Indicaxanthin pre-treatment on their levels was tested. Furthermore, its effect on OECs exposed to A β (1–42), A β (25–35) and A β (35–25) on cell viability, on the production of total Reactive Oxygen Species (ROS), superoxide anion (O₂⁻) and on apoptotic pathway activation were assessed.

Materials And Methods

Materials

Leupeptin, aprotinin, Phenylmethylsulfonyl Fluoride (PMSF), EDTA, EGTA, Sodium Dodecyl Sulfate (SDS), phosphatase inhibitor cocktail II, cytosine arabinoside, full native peptide of A β (1–42), fragment A β (25–35), fragment A β (35–25), 3(4,5-dimethyl-thiazol-2-yl)2,5-diphenyl-tetrazolium bromide (MTT), Dimethyl sulfoxide (DMSO), Lab-Tek II Chamber-Slide Systems, paraformaldehyde and others analytical chemicals were purchased from Sigma-Aldrich (Milan, Italy). Acetic acid and methanol were of LC grade and purchased from Merck (Milan, Italy). Trypsin, antibiotics, heat inactivated Foetal Bovine Serum (GIBCO), Phosphate Buffer Saline solution (PBS), Normal Goat Serum (NGS, GIBCO), Modified Eagle Medium

(MEM) with 2 mM GlutaMAX (GIBCO), Nitrocellulose Membrane Filter Paper Sandwich 0.45 µm pore size (Invitrogen), mouse monoclonal antibody against β-tubulin, anti-rabbit IgG horseradish peroxidase–conjugated and anti-mouse IgG horseradish peroxidase–conjugated were from Thermo Fisher Scientific (Milan, Italy). Bicinchoninic acid method (Pierce/Thermo-Scientific, Rockford, IL). Mini-PROTEAN® TGX™ Precast Protein Gels (4–15%), Mini Trans Blots Filter Paper, 10X Tris/Glycine/SDS buffer, 10X Tris/Glycine buffer, 4x Laemmli Sample Buffer, 2-mercaptoethanol, Precision Plus Protein™ Standard Dual Color, were from Bio-Rad Laboratories Srl (Milan, Italy). Rabbit monoclonal antibody against Cyclin D₁ was from Millipore (Milan, Italy). Mouse monoclonal antibody against TG2 (Neomarkers), mouse monoclonal antibody against Nestin and cellular ROS/Superoxide Detection Assay were from Abcam (Milan, Italy). Mouse monoclonal antibody against GFAP and mouse monoclonal antibody against Vimentin were from DAKO. Mouse monoclonal antibody against caspase-3 was from Becton-Dickinson (Milan, Italy). Cy3 goat anti-mouse and Fluorescein Isothiocyanate (FITC)-conjugated goat anti-mouse IgG antibody were from Jackson Immunological Research Laboratories Inc. Western Lightning Plus-ECL Enhanced Chemiluminescence Substrate was from Perkin-Helmer (Monza, Italy).

Animals

Experiments were carried out on 2-day-old mouse pups (P2), provided by Envigo RMS s.r.l. (Italy). Animals were kept in a controlled environment (23 ± 1°C, 50 ± 5 % humidity) with a 12 h light/dark cycle with food and water available *ad libitum*.

Experiments were carried out in compliance with the Italian law on animal care no. 116 / 1992 and in accordance with the European Community Council Directive (86/609/EEC) and were approved by the Ethical Committee at the University of Catania (Italy). Efforts were made to minimize the number of animals used.

OEC cultures

Olfactory bulbs were removed from decapitated pups and placed in cold (+ 4°C) Leibowitz L-15 medium [27]. Successively, pellets were digested in MEM-H, containing collagenase and trypsin mixture. Trypsinization was stopped by adding DMEM supplemented with 10% FBS (DMEM/FBS). Cells were re-suspended and plated in flasks fed with complete DMEM/FBS. Cytosine arabinoside (10⁻⁵ M), an antimetabolic agent, was added 24 h after initial plating, in order to reduce the number of dividing fibroblasts. OECs were then processed to an additional step transferring from one flask to a new one, in order to reduce contaminating cells, following the method by Chuah and Au [28]. When OECs were confluent, they were removed by trypsin, transferred on 25 cm² flasks and cultured in DMEM/FBS. Cells were then incubated at 37°C in complete medium and fed twice a week. Purified OECs were grown in DMEM/FBS on 14 mm diameter glass coverslips and 96 multiwells flat bottomed at a final density of 1×10⁴ cells/coverslip and on 25 cm² flasks at a final density of 1×10⁶. Cells were then incubated at 37°C a humidified 5% CO₂–95 % air mixture.

Indicaxanthin purification

Indicaxanthin was isolated from *Opuntia ficus indica* L. Mill fruit pulp (yellow cultivar) and purity (97%) of the pigment was assessed by HPLC according to a previous described method [24].

Treatment of OECs

OECs were divided in different groups: a group was stressed for 24 h with of 10 μM A β (1–42) or A β (25–35) or A β (35–25) [18]; another group was treated with 50 μM or 100 μM of Indicaxanthin for 24 h; other two groups were pre-treated with 50 μM or 100 μM of Indicaxanthin for 30 min and subsequently were exposed to 10 μM A β (1–42) or A β (25–35) or A β (35–25). Stock solutions of full native peptide A β (1–42), A β (25–35) and A β (35–25) fragments were diluted in DMSO. For every test, the suitable aliquot from each stock solution was added to culture medium, in order to obtain final concentration 10 μM . A group of cells was treated with a corresponding volume of PBS (final concentration 2% v/v) and used as control. Another group of cell cultures was treated with the corresponding volume of DMSO used to solubilize full native peptide A β (1–42) and A β fragments, having a final DMSO concentration of 2% v/v.

MTT Bioassay

In untreated and treated OECs, 20 μl of 0.5 % MTT solution were added to each multiwell as previous reported [18]. After 2 h of incubation at 37°C, the supernatant was removed, replaced with 200 μl DMSO and incubated at 37°C for 1 h. The optical density of each well sample was measured with a microplate spectrophotometer reader (Titertek Multiskan; Flow Laboratories, Helsinki, Finland) at $\lambda = 570$ nm. On the basis of MTT test, we chose as optimal concentration of Indicaxanthin to treat OECs both in the absence and in the presence of 10 μM A β (1–42), A β (25–35) A β (25–35), 100 μM for 24 h.

Total ROS/O₂⁻ production

In untreated and treated OECs, total ROS and O₂⁻ production were assessed through Cellular ROS/Superoxide Detection Assay, according to manufacturer's instruction. The fluorescent products generated by the two dyes green for total intracellular ROS and orange for O₂⁻ detection were visualized using a wide-field Zeiss fluorescent microscope (Zeiss, Germany) equipped with standard green ($\lambda_{\text{Ex}}/\lambda_{\text{Em}} = 490/525$ nm) and orange ($\lambda_{\text{Ex}}/\lambda_{\text{Em}} = 550/620$ nm) filter set.

Immunocytochemical technique and CLSM analysis

To assess the positivity for Vimentin (proliferation marker), GFAP (differentiation marker), caspase-3 (apoptotic marker), and TG2 in untreated and treated, OECs were processed through immunocytochemical procedures. After 24 h all cells were fixed through 4% paraformaldehyde in 0.1 M PBS for 30 min and then were incubated overnight at 4°C in the following primary antibodies: mouse monoclonal antibody against Vimentin (1:50), mouse monoclonal antibody against GFAP (diluted 1:1000), mouse monoclonal antibody against Nestin (1:200), mouse monoclonal antibody against Caspase-3 (1:500) and mouse monoclonal antibody against TG2 (1:200). FITC anti-mouse (diluted 1:200) and Cy3 anti-mouse (diluted 1:500) were used as secondary antibodies for 1 h at room temperature and in dark condition. Successively, coverslips were washed in PBS and mounted with

PBS/glycerol. The immunostained coverslips were analysed on a Zeiss fluorescent microscope (Zeiss, Germany) and images were captured with an Axiovision Imaging System for GFAP, Nestin, Vimentin and caspase-3. The immunostained for TG2 was obtained using a Confocal Laser Scanning Microscope (CLSM) 510 Meta (Zeiss, Germany), using a X63 lens and captured with an Axiovision Imaging System [6, 18, 29]. The positive labeled cells were counted in ten different microscopic fields (20X magnification) and the positivity for each marker was expressed as percentage and compared with each respective control. No non-specific staining of OECs was observed in control incubations in which the primary antibodies were omitted. To analyze TG2 positive cells Confocal Laser Scanning Microscope (CLSM, LSM-510 Meta, Zeiss, Germany) was used. For the acquisition with CLSM, we used an Apo 63 x/1.4 oil immersion objective and the Argon ($\lambda = 488 \text{ nm}$) and HeNe ($\lambda = 543 \text{ nm}$) lasers. Images were acquired at the pixel resolution of 1024×1024 and were processed to enhance brightness and contrast using the software ZEN 2009. The version number for software ZEN 2009 was 5.5.0.452 and provided together ZEISS confocal microscope. The ZEN 2009 soft version is available at link <https://www.softpedia.com/get/Multimedia/Graphic/Graphic-Viewers/ZEN-2009-Light-Edition.shtml>. The optical fields were examined through green fluorophore excitation.

Isolation of total protein and Western blot analysis

Untreated and treated OECs cells were harvested in cold PBS, collected by centrifugation, resuspended in cell lysis buffer containing 50 mM Tris-HCl (pH 6.8), 150 mM NaCl, 1 mM EDTA, 0.1 mM PMSF, 10 $\mu\text{g}/\text{mL}$ of aprotinin, leupeptin, pepstatin, incubated for 30 min at 4°C , centrifuged at $12,000 \times g$ for 10 min at 4°C and the supernatants containing total cell proteins were collected [5, 18, 30, 31].

Briefly, extracted proteins were stored at -80°C , and protein quantitation was performed by bicinconinic acid method, according to to manufacturer's instruction.

40 μg of total proteins were separated through 4–15% precast SDS–polyacrylamide gels and transferred to nitrocellulose membranes. Filters obtained were then incubated with the following 1:1000 diluted antibodies: mouse monoclonal antibody against TG2, rabbit monoclonal antibody against Cyclin D_1 , mouse monoclonal antibody against β -tubulin. Anti-rabbit IgG horseradish peroxidase–conjugated and anti-mouse IgG horseradish peroxidase–conjugated were then used. The expression of each protein was visualized through Western Lightning Plus-ECL Enhanced Chemiluminescence Substrate after autoradiography filter exposure. Blots were then scanned and quantified through ChemiDoc Imaging System (ChemiDoc™ Imaging System, Bio-Rad, Milan). Densitometric analysis was performed through the integrated software and data obtained were normalized with β -tubulin.

Statistical analysis

Data were statistically analysed using one-way analysis of variance (one-way ANOVA) followed by post hoc Holm–Sidak test to calculate significant differences among groups.

Data were reported represent the mean \pm S.D. of five separated experiments in triplicate, and differences among groups were considered to be significant at $*p < 0.05$.

Results

Cell viability

To monitor cell viability in OECs unexposed and exposed to full native peptide A β (1–42) or to the fragments A β (25–35) and A β (35–25) [18], both in the absence and in the presence of Indicaxanthin, MTT test was performed. In previous our studies we showed that the optimal concentration of A β (1–42), A β (25–35) and A β (35–25) was 10 μ M for 24 h [18]. No significant differences between PBS and DMSO-treated OECs were observed, thus they were used as controls. A significant decrease in the percentage of cell viability in OECs exposed to 10 μ M A β (1–42) or A β (25–35) was found, when compared with controls (Fig. 1). The reverse sequence of A β (35–25) did not show any effect on cell viability, when compared with the controls (Fig. 1). No significant changes in the percentage cell viability were observed in OECs exposed to 50 μ M (Fig. 1B) and 100 μ M (Fig. 1C) of Indicaxanthin, when compared with the controls (Fig. 1A). The pre-treatment of OECs with 50 μ M and 100 μ M of Indicaxanthin and the subsequent exposure to 10 μ M A β (1–42) or A β (25–35) for 24 h was able to restore cell viability to the levels observed in the controls (Fig. 1B, C). Indicaxanthin pre-treatment did not induce any significant changes in OECs exposed to A β (35–25) fragment.

Thus, for the following studies OECs were pre-treated with 100 μ M of Indicaxanthin for 30 min and subsequently exposed to native peptide 10 μ M A β (1–42) or to the fragments A β (25–35) or A β (35–25) for 24 h.

Vimentin and GFAP immunolabeling

To identify glial reactivity after the treatment of OECs exposed to the full native peptide A β (1–42) or to A β (25–35) or A β (35–25) fragments both in the absence and in the presence of Indicaxanthin, we performed immunostaining using antibodies against cytoskeletal proteins Vimentin and GFAP. The exposure of the cells to A β (1–42) or A β (25–35) was able to induce a notable increase in the number of cells positive to Vimentin (Fig. 2) and GFAP (Fig. 3), when compared with the control cultures and those treated with the fragment A β (35–35). In particular, the effect appeared more evident in A β (25–35) exposed OECs, when compared with A β (1–42) ones. The treatment of cultures with 100 μ M of Indicaxanthin did not induce changes for the positivity of the cells for Vimentin and GFAP, when compared with the controls. When the cells were pre-treated with Indicaxanthin and then exposed to A β (1–42) or A β (25–35), the cell positivity for Vimentin and GFAP appeared similar to those observed in control cultures. No specific staining of OECs was observed in control incubation in which the primary antibodies were omitted. This group of experiments demonstrated that full native peptide A β (1–42) and fragment A β (25–35) induced glial activation and that Indicaxanthin pre-treatment was able to counteract it.

Total ROS/O₂⁻ generation

To monitor the intracellular oxidative status, the staining of total intracellular ROS levels (Fig. 4A, green) and O_2^- (Fig. 4B, red) generation in OECs exposed for 24 h to the full native peptide $A\beta(1-42)$ or to the fragment $A\beta(25-35)$ or to $A\beta(35-25)$ both in the absence and in the presence of Indicaxanthin, was assessed. $A\beta$ treatment induced a significant increase in total ROS and O_2^- levels, when compared with the controls. No significant change in total ROS and O_2^- production in $A\beta(35-25)$ and Indicaxanthin alone exposed cells was found. The OEC pre-treatment with Indicaxanthin and the subsequently exposure to $A\beta(1-42)$ or to $A\beta(25-35)$ or to $A\beta(35-25)$ induced a strong reduction both of total intracellular ROS and O_2^- levels. These findings highlighted that $A\beta(1-42)$ and $A\beta(25-35)$ increased the levels of total ROS and O_2^- production and that Indicaxanthin pre-treatment was able to restore the oxidative status modified by $A\beta$ to control values, reducing prevalently O_2^- generation.

Total TG2 expression through immunocytochemistry

The Fig. 5 reports TG2 positivity and its localization performed on single cell through immunocytochemical procedures and CLSM analysis. In the control cells a low staining for TG2 was found and the protein was prevalently localized in the cytosol. A more intense staining for TG2 both in $A\beta(1-42)$ and $A\beta(25-35)$ treated OECs was observed widely in the cytosol, when compared with the controls. In particular, the positivity of the cells for TG2 appeared more evident in $A\beta(25-35)$ treated ones, when compared with the controls and $A\beta(1-42)$ treated OECs. The exposure to the fragment $A\beta(35-25)$ produced a light increase for TG2 cell positivity, when compared with the controls and the protein appeared prevalently localized in the cytosol. In 100 μ M Indicaxanthin-treated cells, TG2 staining was slightly higher than controls and it was widely localized in the cytosol. In contrast, in the cells exposed to $A\beta(1-42)$ and $A\beta(25-35)$, Indicaxanthin pre-treatment was able to decrease the number of positive cells for TG2, when compared with the controls. Specifically, in Indicaxanthin treated OECs and subsequently exposed to $A\beta(1-42)$, TG2 was localized into the nucleus. Differently in $A\beta(25-35)$ -Indicaxanthin pre-treated cells, the protein was prevalently localized in the cytosol, even if some cells showed a low positivity for the protein in the nucleus and nucleoli. A low staining for TG2 in Indicaxanthin pre-treated OECs and then exposed to the fragment $A\beta(35-25)$ was found, when compared both with the controls and the fragment $A\beta(35-25)$ alone. In addition, the protein was prevalently localized in the cytosol. No specific staining of OECs was observed in control incubation in which the primary antibody was omitted.

TG2 and its isoforms expression and effect of Indicaxanthin

To assess and better clarify CLMS analysis performed on single cell relative to the different intracellular localization of TG2, the expression levels of the total TG2 and its isoforms (TG2-S and TG2-L) induced by the different treatment types, were evaluated through Western blot analysis on total cellular lysates. Immunoblots (Fig. 6A) and densitometric analysis (Fig. 6B) show an significant increase of total TG2 expression levels both in $A\beta(1-42)$ and $A\beta(25-35)$ exposed OECs, when compared with the controls. The effect was more evident in $A\beta(25-35)$ -treated cells. Slight but no significant changes in total TG2 expression levels in cultures exposed to $A\beta(35-25)$ were found. Indicaxanthin pre-treatment did not

induce significant modifications in total TG2 expression levels, when compared with the controls. When Indicaxanthin was added to OECs following exposed to A β (1–42) or A β (25–35) or A β (35–25), a significant reduction of total TG2 expression levels was observed, when compared with the controls. The effect of the pre-treatment of the cells with Indicaxanthin was more evident in OECs exposed to A β (25–35). In Indicaxanthin pre-treated OECs and then exposed to the fragment A β (35–25), a significant reduction of total TG2 expression levels was relieved, when compared with A β (35–25), Indicaxanthin alone treated cells and with the controls.

To better elucidate the effect of OEC exposure to A β (1–42) or A β (25–35) or A β (35–25) both in the absence and in the presence of Indicaxanthin on the role played by TG2, the expression levels of its isoforms were detected. Figure 7 reports the immunoblots (Fig. 7A) and densitometric analysis (Fig. 7B, C) performed on all experimental conditions. On the basis of the treatment type, different expression patterns of the isoforms were found. In controls (PBS and DMSO) both TG2 isoforms were expressed at very low levels, even if TG2-S levels were higher than TG2-L. A β (1–42) treatment induced a significant increase of both isoform expression levels, when compared with the controls. The exposure of OECs to A β (25–35) caused a significant increase of TG2-S and TG2-L, even if TG2-S levels were higher than TG2-L ones, when compared with A β (1–42) exposed cells and with the controls. No significant change in TG2-L and TG2-S expression levels in cultures exposed to A β (35–25) was found. When OECs were exposed to 100 μ M of Indicaxanthin, a significant increase of the both isoforms was found, when compared with the controls. The pre-treatment of the cells with Indicaxanthin and A β (1–42) exposure induced a significant increase of TG2-L, when compared with TG2-S and with the controls. In contrast, Indicaxanthin pre-treatment in A β (25–35) exposed cells caused a significant enhancement of TG2-S expression levels, when compared with TG2-L ones. Furthermore, no significant changes in TG2-L expression levels in A β (35–25) exposed OECs were observed. Surprisingly, Indicaxanthin pre-treatment caused a significant increase of the TG2-S expression levels, when compared with A β (35–25), Indicaxanthin alone treated cells and with the controls. Densitometric analysis performed for each experimental conditions, after normalization with β -tubulin, confirmed all the results. These data highlighted that A β treatment both in the absence and in the presence of Indicaxanthin differently modulates TG2 isoforms acting on apoptotic pathway activation or on the cell self-renewal ability.

Caspase-3 cleavage immunolabeling

To verify the TG2-mediated apoptotic pathway in OECs exposed to A β (1–42) or A β (25–35), we evaluated the caspase-3 cleavage through immunocytochemical techniques. Figure 8 highlights caspase-3-positive OECs exposed to different conditions. In controls (PBS and DMSO) the positivity of cells for caspase-3 was almost absent. When the cells were exposed to A β (1–42) or A β (25–35), a strong activation of positive cells for caspase-3 was found, which appeared mainly localized in the cytoplasm. The effect was particularly evident in A β (25–35), that is highly toxic for the cells. In A β (35–25)-treated cells a light positivity for caspase-3, when compared with the controls, was found. The treatment of OECs with 100 μ M of Indicaxanthin did not produce any positivity for caspase-3, when compared with the controls. In Indicaxanthin pre-treated cells and subsequently exposed to A β (1–42) or A β (25–35) a decrease for

caspase-3-positive cells, which appeared at similar expression levels of the controls, was observed. A light increase of positive OECs was found in Indicaxanthin pre-treated cells and then exposed to the fragment A β (35–25), when compared with Indicaxanthin alone and with the controls. No specific staining of OECs was observed in control incubation in which the primary antibody was omitted. These findings revealed the role played by TG2 in the control of apoptotic pathway activation both in A β exposed OECs and Indicaxanthin pre-treated ones.

Cyclin D₁ expression levels and Nestin immunolabeling

To assess the role played of TG2 in the cellular repair induced by Indicaxanthin on OEC exposed to A β , cyclin D₁ expression levels and the cell positivity for Nestin were examined. Western Blot and densitometric analysis for cyclin D₁ performed on total cellular lysates from A β (1–42), A β (25–35) and A β (35–25) exposed OECs both in the absence and in the presence of 100 μ M Indicaxanthin were reported in the Fig. 9A,B. A significant decrease in cyclin D₁ expression levels in A β (1–42) exposed cells, when compared with controls, were found. The toxic fragment A β (25–35) induced very strong reduction of cyclin D₁ expression levels respect to the full native A β (1–42) peptide and the controls. In contrast, the exposure of OECs to no-toxic fragment A β (35–25) did not cause significant modifications in cyclin D₁ expression levels, when compared with the controls. The pre-treatment of the cells with Indicaxanthin alone did not induced any significant change of protein expression levels, when compared with the controls. When it was added to OECs following exposed to A β (1–42), a significant increase of cyclin D₁ expression levels, when compared with A β (1–42) alone was observed, and protein levels appeared similar to those found in the controls and in Indicaxanthin alone. A significant increase of cyclin D₁ expression levels in A β (25–35) treated with Indicaxanthin was highlighted, when compared with A β (25–35) alone (Fig. 9A, B), even if its levels were at lower than Indicaxanthin plus A β (1–42), Indicaxanthin alone and controls.

Figure 10 reports the positivity for Nestin in OECs exposed to Indicaxanthin in the absence and in the presence of A β (1–42), A β (25–35), A β (35–25). A significant reduction in the number of positive cells for Nestin in A β (1–42) and A β (25–35) exposed was found, when compared with the controls. A β (35–25) exposed cells showed a light increase for Nestin positive OECs, when compared with the controls. The pre-treatment of cultures with 100 μ M of Indicaxanthin for 24 h did not induce changes for Nestin positive cells, which appeared similar to the levels observed in the controls. When OECs were pre-treated with 100 μ M of Indicaxanthin and subsequently were exposed to A β (1–42) or A β (25–35), a strong increase of positive cells for Nestin was found, when compared with the controls. A low of Nestin positivity in Indicaxanthin pre-treated cells and then exposed to A β (35–35) was shown. No specific staining of OECs was observed in control incubation in which the primary antibody to was omitted.

This set of experiments demonstrated that Indicaxanthin pre-treatment stimulated TG2 repair activity in OEC exposed to A β , activating also the stem self-renewal through the increase of cyclin D₁ expression levels and the cell positivity for Nestin.

Discussion

The aim of this study was to assess TG2 and its isoform expression levels in both OECs exposed to full native peptide of A β (1–42) and its toxic fragment A β (25–35). Epidemiological evidences report that the effects of Mediterranean Diet “MeDi” could be an alternative prophylaxis treatment for AD [32]. In particular, it has been identified in Sicily an increased frequency of centenarians, a reduced occurrence of mental and cognitive diseases, when compared with other Italian or European regions [19, 20]. One of the factors that could contribute to this phenomenon is the large availability of some rare specific nutrients, largely present in some area of Sicily, as well as Indicaxanthin from *Opuntia ficus-indica* fruit. Therefore, for the first time, we tested the effect of Indicaxanthin pre-treatment on OECs exposed to A β . Since cytoskeleton plays an important role in the pathogenesis of neurodegenerative diseases, including AD [33], particular attention was focused on the effect of Indicaxanthin on some cytoskeletal proteins, such as Vimentin, GFAP, that have an important role in astrogliosis, a typical sign of AD [34]. Furthermore, the expression levels of cyclin D₁, which is induced in stem cell reprogramming and is co-expressed with Nestin, marker of neural stem cells [26], were assessed. In addition, the effect of A β (1–42), A β (25–35) and A β (35–25) in the absence and in the presence of Indicaxanthin was tested on cellular viability and on the activation of apoptotic pathway. Intracellular total ROS and O₂⁻ production was also evaluated. The experiments were performed on OECs because they represent a glial population of olfactory system that is also involved in AD [12]. It is note that olfactory dysfunction, as well as hyposmia and olfactory memory loss, represent the early symptoms of AD [18, 35, 36]. Furthermore, it has been demonstrated that anterior olfactory nucleus (AON) projects to hippocampus [37] and that it is the earliest site involved in AD, associated with cell loss, the neurofibrillary tangles and senile plaques [12].

Previous our researches demonstrated that TG2 is overexpressed in OECs exposed to A β (1–42) and its toxic fragment A β (25–35) and that the treatment with some Growth Factors (GFs) was able to restore its levels to control values [18]. In particular, TG2, a calcium-dependent protein with transamidating activity, is involved in AD, inducing the formation of insoluble amyloid aggregates that can alter the properties of several proteins [2]. TG2 activity is down-regulated in response to oxidative stress [29, 30] and this effect could be related to the increase of the intracellular Ca²⁺ levels due to A β toxicity [18]. In fact, the accumulation of extracellular protein aggregates prevalently constituted by polymeric A β , caused by the aberrant transamidating activity of TG2, are also related to a dysregulation of autophagy process [38]. These conditions contribute to oxidative stress and neural cell death, in which TG2 plays a key role [30]. It has been reported that hippocampal neurons are more responsive to Indicaxanthin [39]. In particular, it has an important role in several metabolic functions both *in vitro* and *in vivo*, reducing inflammation and enhancing immune response [22, 23, 40].

In this study, for the first time, we highlight that the OEC exposure to A β (1–42), its fragments A β (25–35) and A β (35–25) induces a different expression pattern of TG2-L and TG2-S, demonstrating the opposite role played by TG2. Furthermore, we show the protective effect exerted by Indicaxanthin pre-treatment on total TG2 and its isoforms expression levels. In particular, we found that in A β (1–42) treated cells the two isoforms appeared at same expression levels, whereas in A β (25–35) exposed ones TG2-S was at higher

levels than TG2-L, when compared with A β (1–42) exposed cells and with the controls. In OECs exposed to A β (35 – 25), a light modification between TG2-L and TG2-S expression levels was observed. The pre-treatment with Indicaxanthin was able to counteract the oxidative damage following the exposure of the cells to full native peptide of A β (1–42) and its toxic fragment A β (25–35), restoring the expression levels of total TG2 to control values. Furthermore, CLSM analysis performed on single cell showed that TG2 in OECs pre-treated with Indicaxanthin alone was localized in the cytosol. In contrast, when cells were pre-treated with Indicaxanthin and then exposed to A β (1–42), the protein appeared prevalently localized into the nuclear compartment. In the cells pre-treated with Indicaxanthin and then stressed with A β (25–35), TG2 was localized both in the cytosol and in the nucleus. Western blot analysis showed a significant increase in TG2-L in Indicaxanthin alone treated cells and in those then exposed cells to A β (1–42). This effect might be correlated to the role played by TG2 when it is localized into the nuclear compartment, in which it acts on the control of cell proliferation, regulating gene expression, cell survival and differentiation, exerting an anti-apoptotic function [10, 18, 41]. In OECs treated with Indicaxanthin alone and in those subsequently exposed to A β (25–35), an increase of TG2-S expression levels was observed. The effect appeared more evident in the cells pre-treated with Indicaxanthin. TG2-S, even if reduced respect to that found in A β (25–35) treated cells, exerts transamidating activity and acts as apoptotic factor [6, 18]. Surprisingly, Indicaxanthin pre-treatment in A β (35 – 25) exposed cells, induced a significant increase of TG2-S expression levels, when compared with A β (35 – 25) alone and controls. This finding might be due to the strong protective effect of Indicaxanthin, since we hypothesize that A β (35 – 25) fragment, even if it was reported that is not toxic [25], was able to induce a low toxicity in OECs, as relieved by a very significant increase of TG2-S expression levels when compared with exposed cells to A β (35 – 25) alone. Thus, we suppose that this effect may be due to the protective role played by TG2, which stimulates its pro-apoptotic activity, in order to remove damaged cells and to induce cellular repair. We also found that Indicaxanthin counteracted the oxidative stress induced by A β , as relieved by the reduction of total ROS and O $_2^-$ production, that appeared similar to those observed in the controls. Thus, Indicaxanthin pre-treatment, for its antioxidant properties, was able to reduce the A β -toxicity, oxidative stress-dependent and mitochondrial damage. In addition, Indicaxanthin, with its anti-inflammatory properties, decreased GFAP and Vimentin expression levels, that were enhanced in A β exposed OECs. These results highlighted that Indicaxanthin exerted a protective effect on reactive astrogliosis induced by A β responsive of cytoskeleton modifications. Furthermore, to clarify the protective role played by TG2 in the absence and in the presence of Indicaxanthin, the levels of Nestin, marker of neural stem self-renewal, co-expressed with cyclin D $_1$, marker of cellular proliferation [26], were assessed. These results show an increase of positive cells for Nestin and cyclin D $_1$ expression levels, demonstrating that Indicaxanthin pre-treatment, stimulating the activity played by nuclear TG2 on stem self-renewal OEC reprogramming, that stimulates cell proliferation repairing the damage induced by A β . We also observed that Indicaxanthin counteracted the TG2-aberrant cross-linking activity induced by A β -exposure on the cells, evaluating caspase-3 cleavage, that appeared reduced following to its treatment. This effect might be correlated to the function that TG2 exerts on the apoptotic pathway, as revealed by the increase of TG2-S expression levels observed in our experimental conditions, when cells were treated with A β (1–42) and A β (25–35) in the absence of Indicaxanthin. In contrast, total TG2 did not show its opposite role on the

basis of cellular localization and did not evidence the effect of A β both in the absence and in the presence of Indicaxanthin.

Taken together, our findings demonstrate that A β stress is responsible of TG2 up-regulation [18] and its structural modifications in two distinct conformational states with different functions [10]. In fact, when the levels of Ca²⁺ are low and those of guanosin triphosphate (GTP) or guanosin diphosphate (GDP) are high, TG2-L acts as a GTPase, is involved in signaling pathway, is inactive and is present in “closed” conformation, promoting cell growth and survival (Fig. 11A). A β exposure of OECs, increasing intracellular Ca²⁺ and decreasing GTP or GDP levels, might cause a change of TG2-L from “closed” to “open” conformation, catalytically active. In addition, A β treatment induced an increase of the levels of TG2-S, an alternative splice variant of TG2 lacking of the portion of the carboxyl terminal essential for the maintenance of the protein in the “closed” conformation, that is responsible of apoptotic activation and cell death. The effect is more evident when the cells were exposed to the major toxic A β (25–35) fragment, that strongly enhanced intracellular Ca²⁺ levels (Fig. 11B). Indicaxanthin pre-treatment prevented total TG2 over-expression induced by the OEC exposure to full native peptide A β (1–42) and A β (25–35) fragment, probably binding to Ca²⁺ [40]. The significant increase of TG2-L isoform expression levels induced by A β (1–42), accompanied by the decrease of TG2-S ones, is related to the role that the protein plays into the nucleus, in which it might stimulate OEC self-renewal and the reparative effect against A β toxicity (Fig. 11C). Furthermore, in A β (25–35) exposed OECs Indicaxanthin is able to significantly decrease TG2-S isoform expression levels enhancing at the same time those of TG2-L. The different expression pattern of TG2 isoforms in A β (25–35) exposed cells in the presence of Indicaxanthin might be due to the major toxicity of the fragment that induces a major enhancement of Ca²⁺. Thus, in this conditions, the protein was able to stimulate both apoptosis and self-renewal (Fig. 11D).

Conclusions

Our findings clearly highlighted that A β exposure on OECs induced an increase of TG2 and a different expression pattern of its isoforms. Furthermore, the pre-treatment of the cells with Indicaxanthin was able to decrease total TG2 expression levels, inducing a different pattern of TG2 isoforms that might be due to change of TG2 state conformation. It also reduced total ROS and O₂⁻ production and the expression levels of GFAP and Vimentin, inhibiting glial reactivity and the activation of apoptotic pathway induced by A β . Furthermore, it leads to an increase of Nestin and cyclin D₁ expression levels, stimulating OECs self-renewal and TG2 reparative role. In addition, our data suggest that in OECs exposed to A β both in the absence and in the presence of Indicaxanthin might differently induce the transition of TG2 between “closed” and “open” conformation providing a new mechanism involved in the signal pathways activated by the protein in A β injury. Therefore, further studies need to better clarify whether Indicaxanthin plays an important role for adopting the TG2 open conformation, that has a key role in self-renewal ability of OECs, which are cells capable of expressing and releasing neurotrophic receptors, and might represent a promising tool for neural regeneration in AD.

Abbreviations

AD: Alzheimer Disease; A β : Amyloid Beta; TG2: Tissue transglutaminase; TG2-L: Tissue transglutaminase Long; TG2-S: Tissue transglutaminase Short; OECs: Olfactory Ensheathing Cells; ROS: Reactive Oxygen Species; O $_2^-$: Superoxide anion; GFAP: Glial Fibrillary Acidic Protein; DMEM: Dulbecco Modified Eagles Medium; FBS: Foetal Bovine Serum.

Declarations

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Authors' contributions

AC and RP: design of the study and writing of the first draft. GS and MS: acquisition and analysis of data. RG: statistical analysis. GR, RG, MAC, UC reviewed the manuscript for important intellectual content. AA, LT, MA purified and synthesized indicaxanthin All authors read and approved the final manuscript.

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Ethics approval and consent to participate

Experiments were carried out in compliance with the Italian law on animal care no. 116 / 1992 and in accordance with the European Community Council Directive (86/609/EEC) and were approved by the Ethical Committee at the University of Catania (Italy).

Consent for publication

All authors have given their consent for publication.

Competing interests

The Authors declare that they have no conflicts of interest.

Availability of data and material

The data used and analysed during the current study are available from the corresponding author on reasonable request.

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Figures

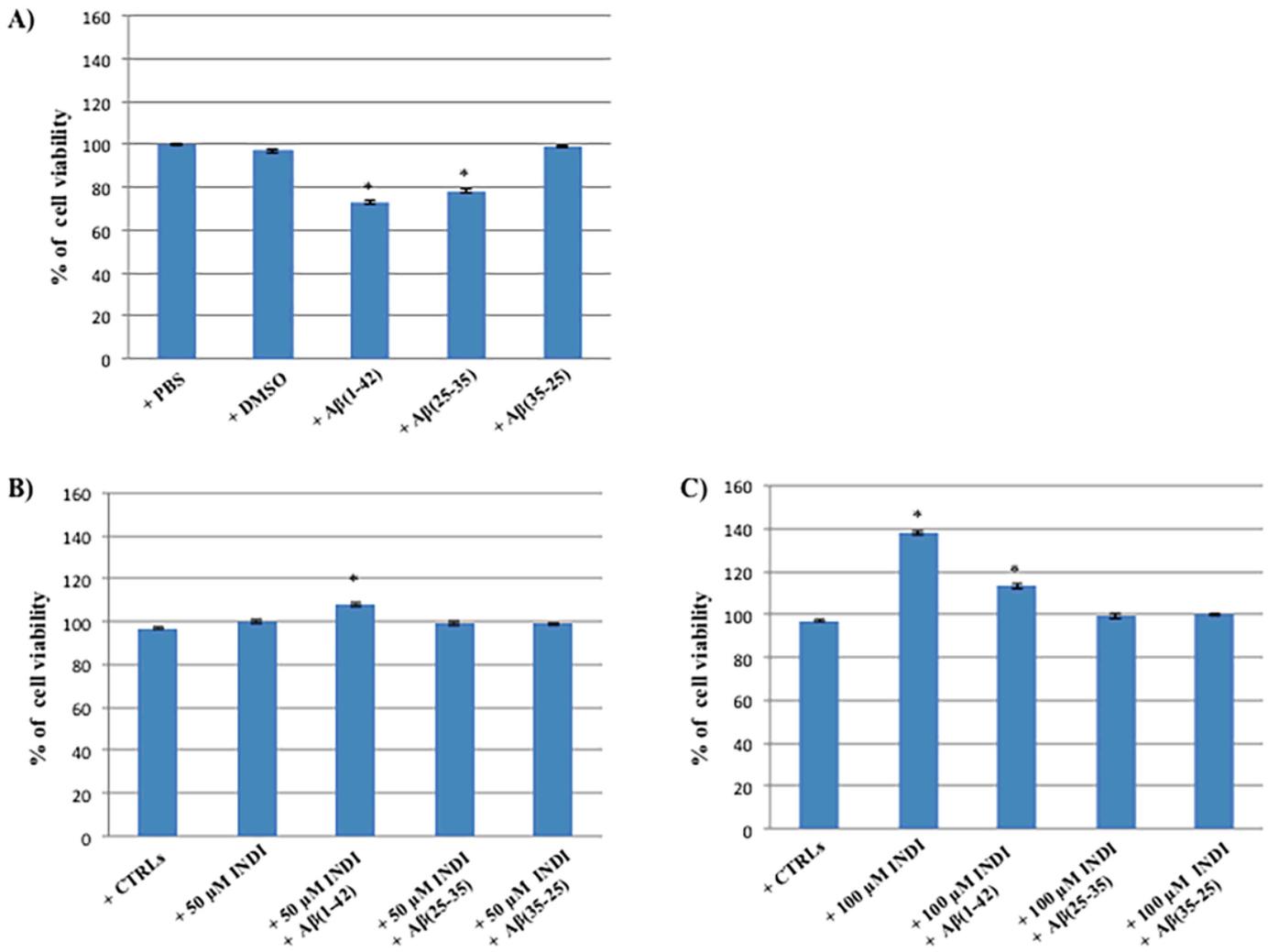


Figure 1

Percentage of cell viability in OECs performed through MTT test. (A) PBS and DMSO treated OECs, exposed to 10 μM A β (1-42) or A β (25-35) or A β (35-25) for 24 h; (B) OECs pre-treated with 50 μM Indicaxanthin (INDI) and exposed to 10 μM A β (1-42) or A β (25-35) or A β (35-25) for 24 h; (C) OECs pre-treated with 100 μM INDI and exposed to 10 μM A β (1-42) or A β (25-35) or A β (35-25) for 24 h. Data were statistically analysed by using one-way analysis of variance (one-way ANOVA) followed by post hoc Holm-Sidak test, in order to calculate significant differences among groups. Data were reported represent the mean \pm S.D. of five separated experiments in triplicate. * $p < 0.05$ significant differences vs controls.

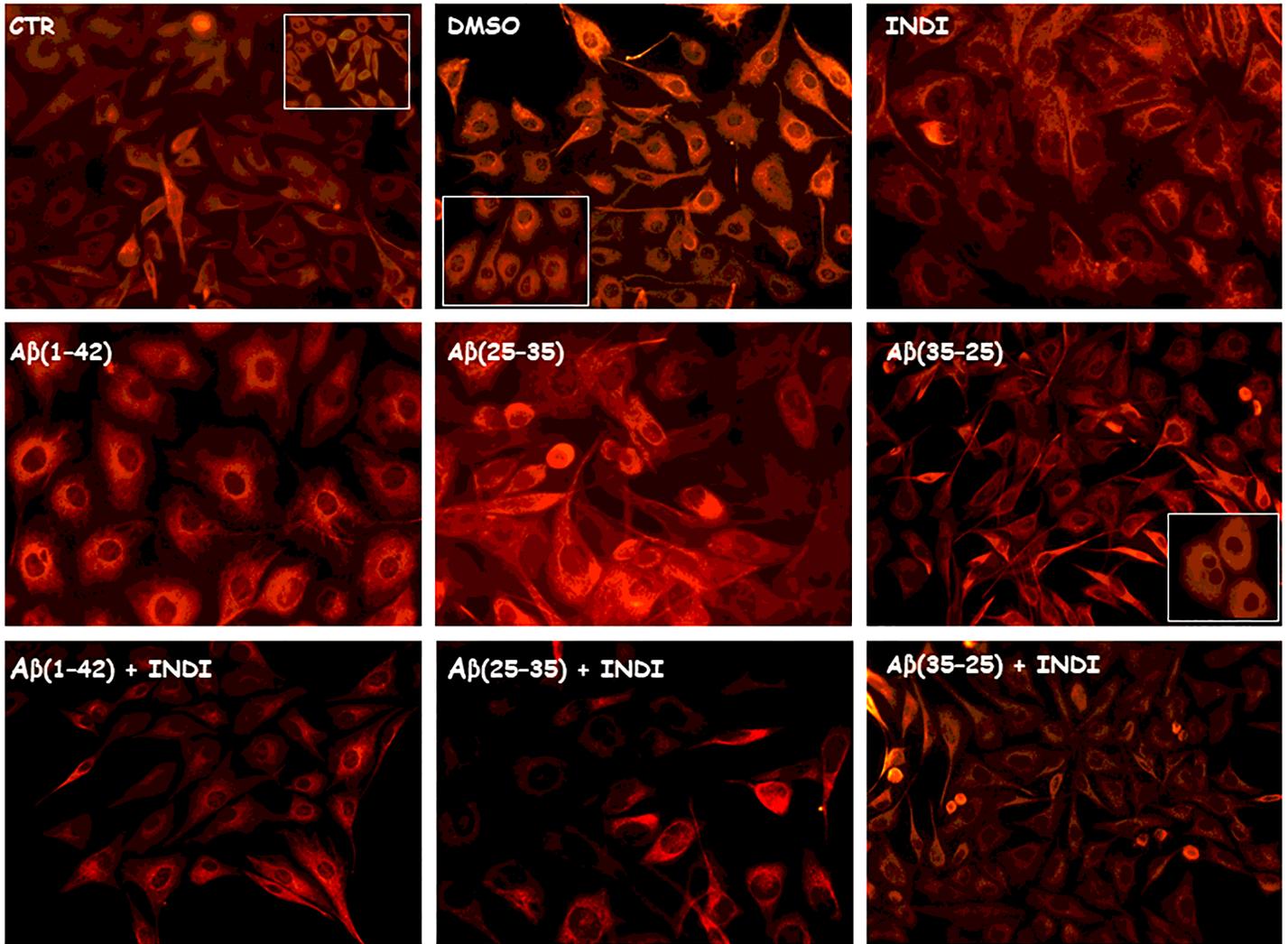


Figure 2

Immunocytochemistry for anti-Vimentin in OECs. Images show different conditions: control, DMSO, 100 μM Indicaxanthin (INDI), 10 μM Aβ(1-42) or Aβ(25-35) or Aβ(35-25) both in the absence and in the presence of 100 μM Indicaxanthin for 24h. Scale bar 20 μm.

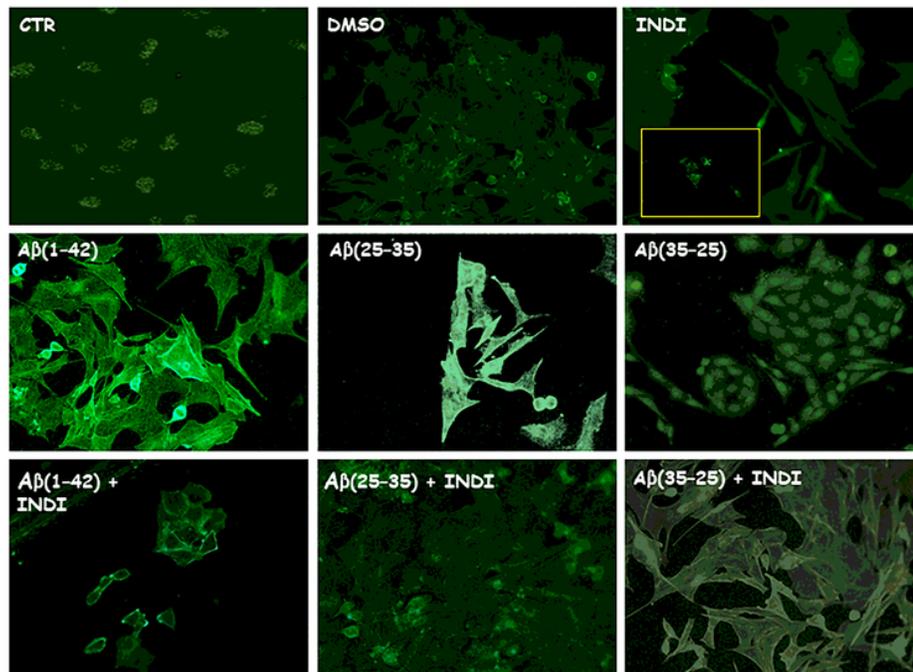


Figure 3

Immunocytochemistry for anti-GFAP in OECs. Images show different conditions: control, DMSO, 100 μ M Indicaxanthin (INDI), 10 μ M A β (1–42) or A β (25–35) or A β (35–25) both in the absence and in the presence of 100 μ M INDI for 24h. Scale bar 20 μ m.

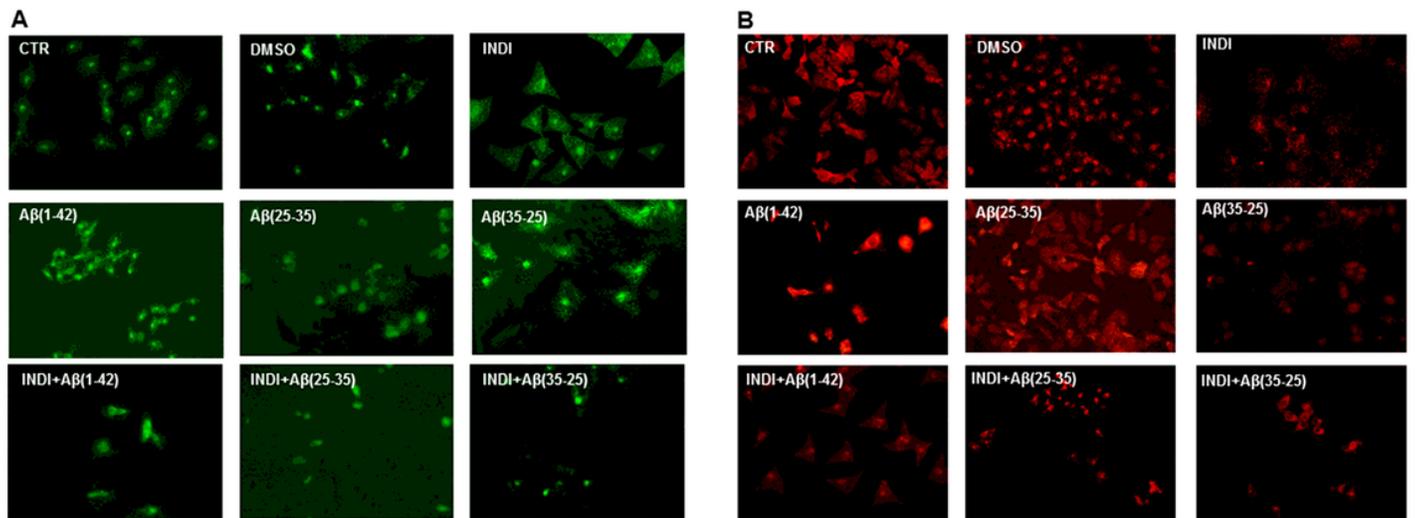


Figure 4

Staining of total intracellular ROS levels and O₂⁻. Total intracellular ROS levels (green, Fig. 4A) and O₂⁻ (red, Fig. 4B) generation in OECs in different conditions: control, DMSO, 100 μM Indicaxanthin (INDI), 10 μM Aβ(1–42) or Aβ(25–35) or Aβ(35–25) both in the absence and in the presence of 100 μM INDI for 24h. Scale bar 20 μm.

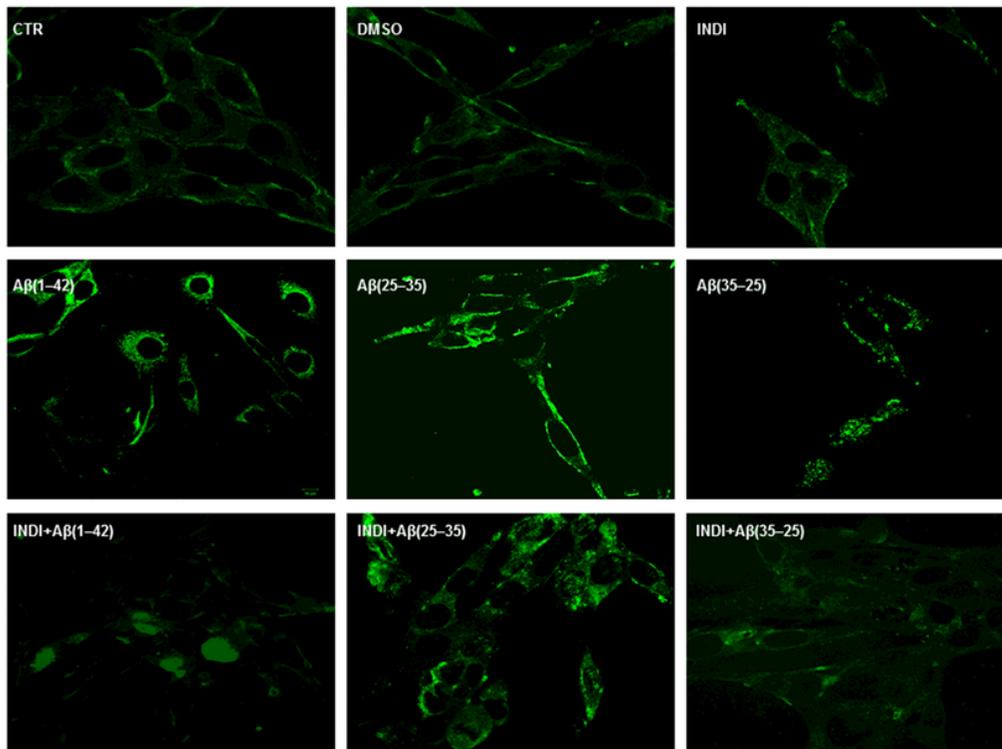


Figure 5

Confocal Laser Scanning Microscopy of labeling immunocytochemistry for anti-TG2 in OECs. Images show different conditions: control, DMSO, 100 μM Indicaxanthin (INDI), 10 μM Aβ(1-42) or Aβ(25-35) or Aβ(35-25) both in the absence and in the presence of 100 μM INDI for 24h. Scale bar 20 μm.

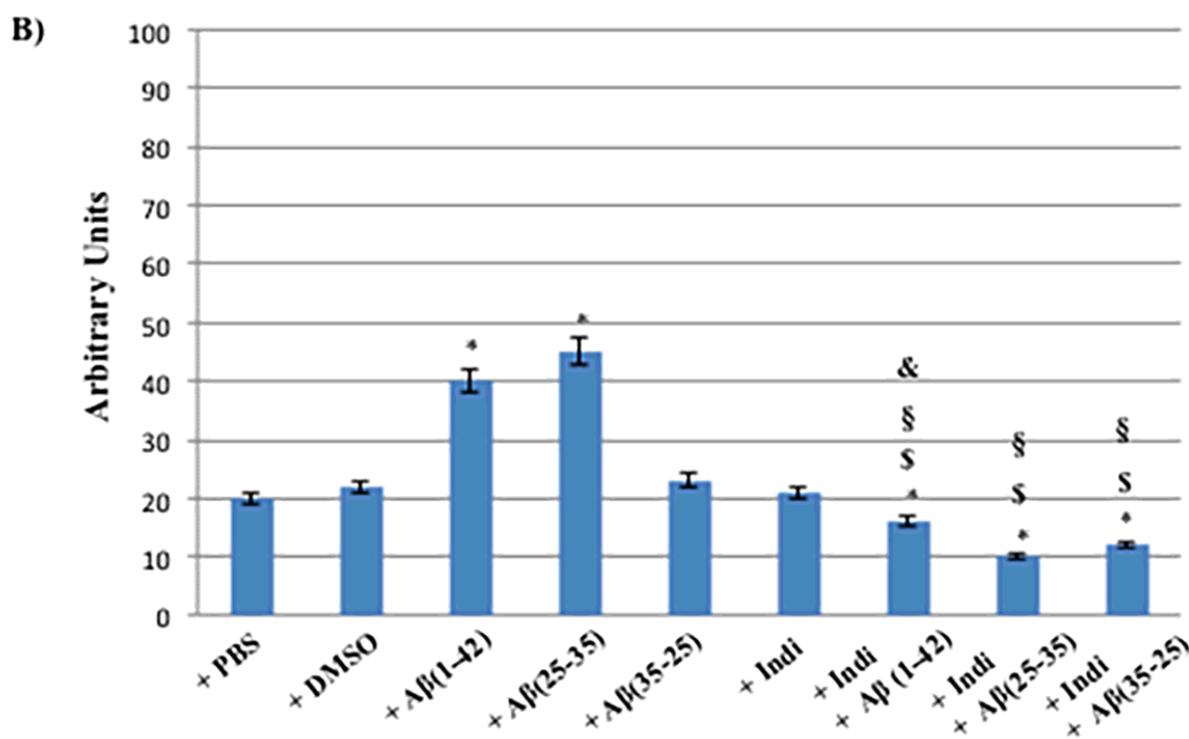
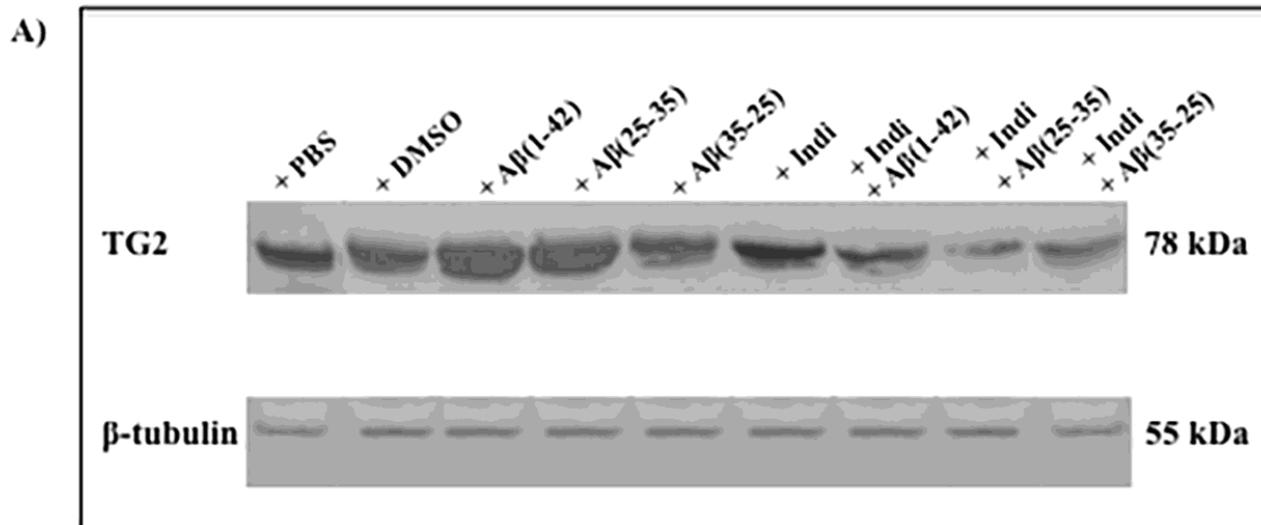


Figure 6

Western Blotting analysis. (A) Representative immunoblot for total TG2 expression levels in total cellular lysates from OECs in different conditions: control, DMSO, 100 μ M Indicaxanthin (INDI), 10 μ M A β (1-42) or A β (25-35) or A β (35-25) both in the absence and in the presence of 100 μ M INDI for 24h. (B) Densitometric analysis of TG2 expression levels performed after normalization with β -tubulin. The results are expressed as the mean \pm S.D. of the values of five separate experiments performed in triplicate. * p <

0.05 significant differences vs controls; \$p < 0.05 significant differences of A β (1-42) + INDI or A β (25-35) + INDI or A β (35-25) + INDI vs INDI; §p < 0.05 significant differences of A β (1-42) + INDI vs A β (1-42), A β (25-35) + INDI vs A β (25-35) or A β (35-25) + INDI vs A β (35-25); &p < 0.05 significant differences of A β (1-42) + INDI vs A β (25-35) + INDI or vs A β (35-25) + INDI.

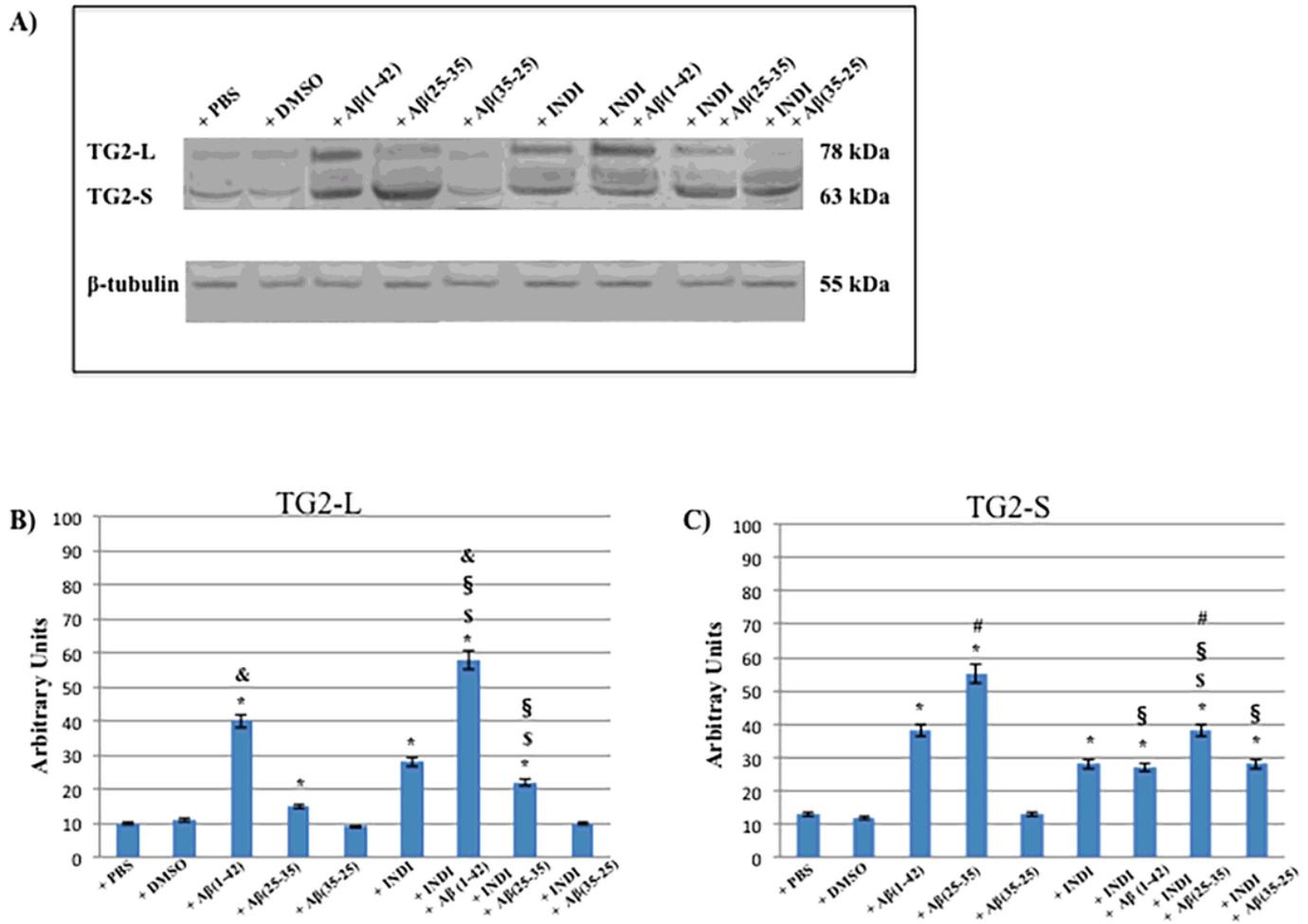


Figure 7

Western Blotting analysis. (A) Representative immunoblots through Western Blotting analysis for TG2 isoforms (TG2-L and TG2-S) expression levels in total cellular lysate from OECs in different conditions: control, DMSO, 100 μ M Indicaxanthin (INDI), 10 μ M A β (1-42) or A β (25-35) or A β (35-25) both in the absence and in the presence of 100 μ M INDI for 24h; (B) Densitometric analysis of TG2-L expression levels performed after normalization with β -Tubulin. (C) Densitometric analysis of TG2-S expression levels performed after normalization with β -tubulin. The results are expressed as the mean \pm S.D. of the values of five separate experiments performed in triplicate. *p < 0.05 significant differences vs controls; \$p < 0.05 significant differences of A β (1-42) + INDI or A β (25-35) + INDI or A β (35-25) + INDI vs INDI; §p < 0.05 significant differences of A β (1-42) + INDI vs A β (1-42), A β (25-35) + INDI vs A β (25-35) or A β (35-25) + INDI vs A β (35-25); &p < 0.05 significant differences of A β (1-42) vs A β (25-35) or vs A β (35-25) and of A β (1-

42) vs $A\beta(25-35)$ + INDI or vs $A\beta(35-25)$ + INDI; # $p < 0.05$ significant differences of $A\beta(25-35)$ vs $A\beta(1-42)$ or vs $A\beta(35-25)$ and of $A\beta(25-35)$ + INDI vs $A\beta(1-42)$ + INDI or vs $A\beta(35-25)$ + INDI.

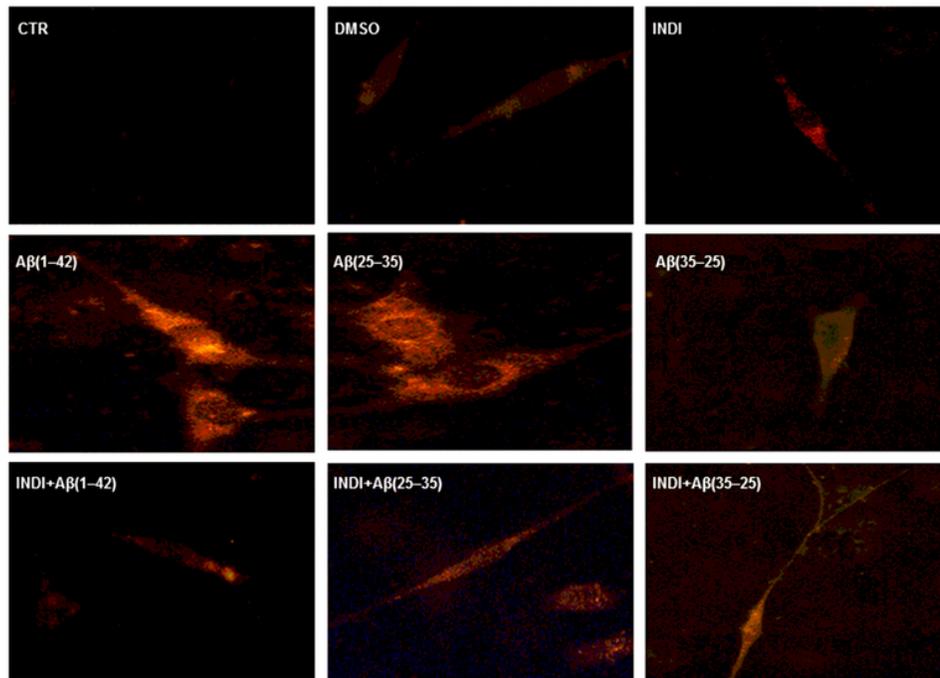


Figure 8

Immunocytochemistry for anti-Caspase-3 in OECs. Images show different conditions: control, DMSO, 100 μ M Indicaxanthin (INDI), 10 μ M $A\beta(1-42)$ or $A\beta(25-35)$ or $A\beta(35-25)$ both in the absence and in the presence of 100 μ M INDI for 24h. Scale bar 20 μ m.

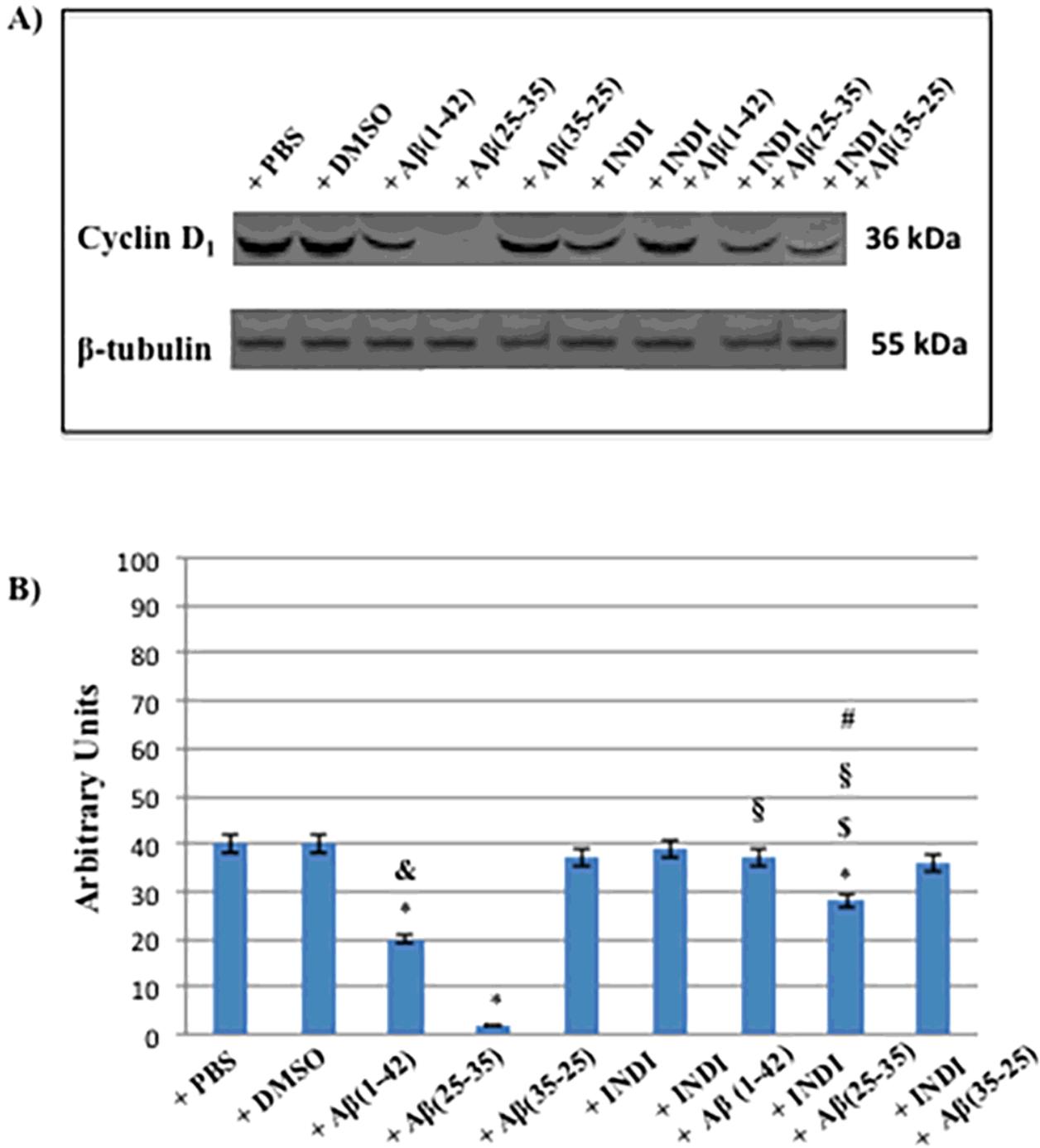


Figure 9

Western Blotting analysis. (A) Representative immunoblot through Western Blotting analysis for cyclin D1 expression levels in total cellular lysates from OECs in different conditions: control, DMSO, 100 μ M Indicaxanthin, 10 μ M A β (1–42) or A β (25–35) or A β (35–25) both in the absence and in the presence of 100 μ M Indicaxanthin for 24h. (B) Densitometric analysis of cyclin D1 expression levels performed after normalization with β -tubulin. The results are expressed as the mean \pm S.D. of the values of five separate

experiments performed in triplicate. * $p < 0.05$ significant differences vs controls. \$ $p < 0.05$ significant differences of $A\beta(1-42) + INDI$ or $A\beta(25-35) + INDI$ or $A\beta(35-25) + INDI$ vs $INDI$; § $p < 0.05$ significant differences of $A\beta(1-42) + INDI$ vs $A\beta(1-42)$, $A\beta(25-35) + INDI$ vs $A\beta(25-35)$ or $A\beta(35-25) + INDI$ vs $A\beta(35-25)$; & $p < 0.05$ significant differences of $A\beta(1-42)$ vs $A\beta(25-35)$ or vs $A\beta(35-25)$; # $p < 0.05$ significant differences of $A\beta(25-35) + INDI$ vs $A\beta(1-42) + INDI$ or vs $A\beta(35-25) + INDI$.

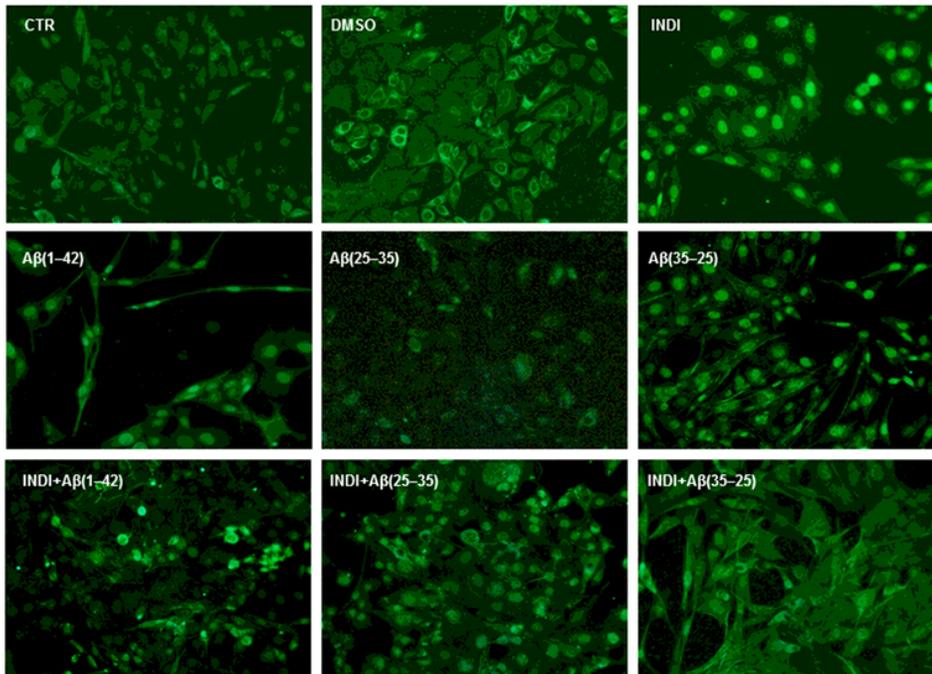


Figure 10

Immunocytochemistry for anti-Nestin in OECs. Images show different conditions: control, DMSO, 100 μM Indicaxanthin (INDI), 10 μM $A\beta(1-42)$ or $A\beta(25-35)$ or $A\beta(35-25)$ both in the absence and in the presence of 100 μM INDI for 24h. Scale bar 20 μm .

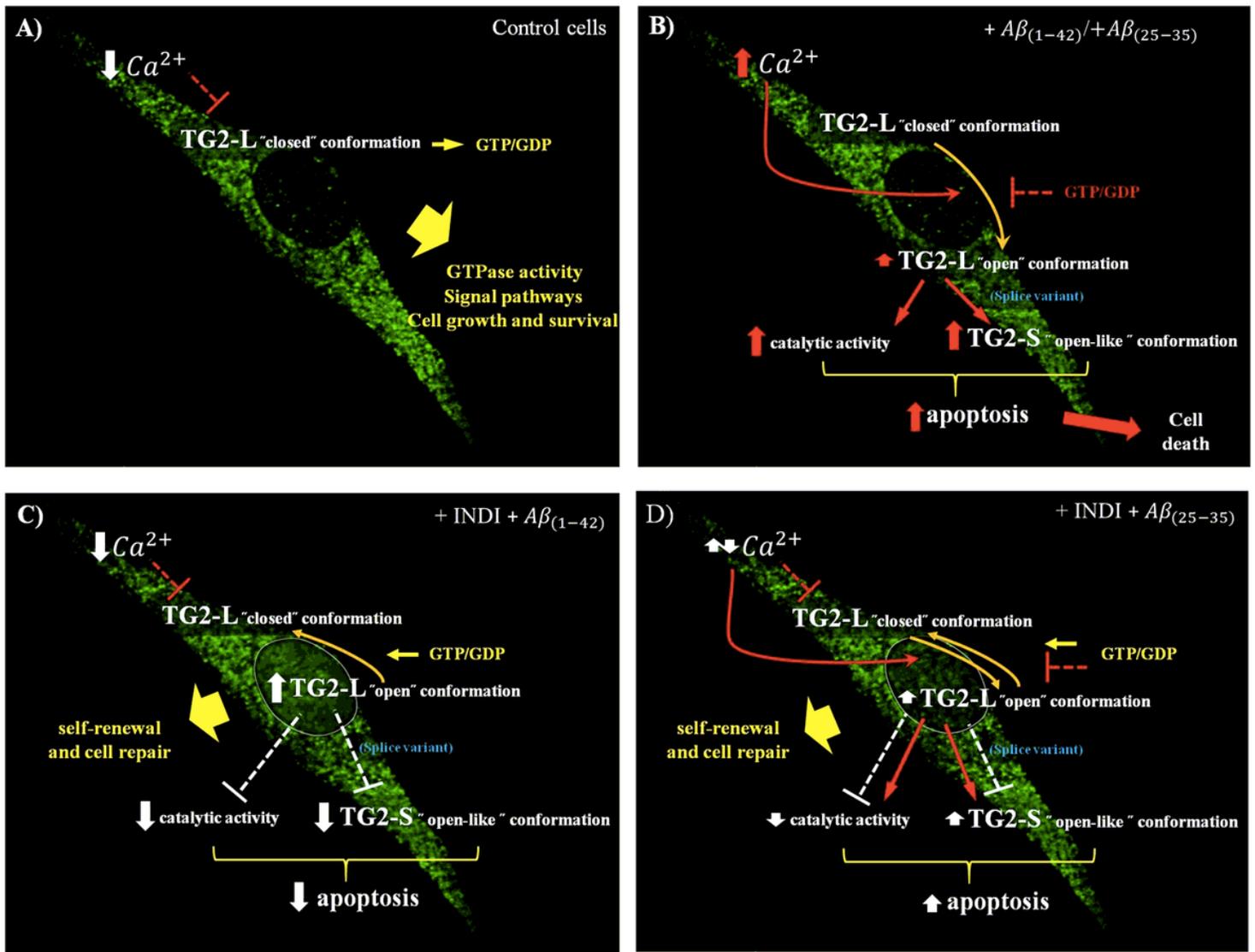


Figure 11

Drawing regarding the effects of OEC treatments with full native peptide $A\beta_{(1-42)}$ and $A\beta_{(25-35)}$ fragment on TG2 and its isoforms (TG2-L and TG2-S) expression levels both in the absence and in the presence of Indicaxanthin (INDI). (A) TG2-L and TG2-S expression levels in OECs maintained in normal conditions; (B) TG2-L and TG2-S expression levels in OECs exposed to 10 μ M $A\beta_{(1-42)}$ or $A\beta_{(25-35)}$ for 24 h; (C) TG2-L and TG2-S expression levels in OECs pre-treated with 100 μ M of INDI and exposed to 10 μ M $A\beta_{(1-42)}$ for 24 h; (D) TG2-L and TG2-S expression levels in OECs pre-treated with 100 μ M of INDI and exposed to 10 μ M $A\beta_{(25-35)}$ for 24 h.