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SHORT REPORT/RAPID COMMUNICATION

PDGF-BB Protects Mitochondria from Rotenone in T98G Cells

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Abstract Rotenone is one of the most-studied neurotoxic substances as it induces oxidative stress processes both in cellular and animal models. Rotenone affects ATP generation, reactive oxygen species (ROS) production, and mitochondrial membrane potential in neurons and astrocyte-like cells. Previous epidemiologic studies have supported the role of neurotrophic factors such as BDNF and GDNF in neuroprotection mainly in neurons; however, only very few studies have focused on the importance of astrocytic protection in neurodegenerative models. In the present study, we assessed the neuroprotective effects of PDGF-BB against toxicity induced by rotenone in the astrocytic-like model of T98G human glioblastoma cell line. Our results demonstrated that pretreatment with PDGF-BB for 24 h increased cell viability, preserved nuclear morphology and mitochondrial membrane potential following stimulation with rotenone, and reduced ROS production nearly to control conditions. These observations were accompanied by important morphological changes induced by rotenone and that PDGF-BB was able to preserve cellular morphology under this toxic stimuli. These findings indicated that PDGF-BB protects mitochondrial functions, and may serve as a potential therapeutic strategy in rotenone-induced oxidative damage in astrocytes.

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Introduction

Rotenone is one of the most-studied neurotoxic substances used as a model for Parkinson disease (PD) and oxidative stress events in cellular and animal models (Betarbet et al. 2000; Greenamyre et al. 2003). This neurotoxin is a naturally occurring isoflavonoid produced in the leaves, roots, and rhizomes of tropical legumes from the genres *Derris*, *Lonchocarpus*, and *Tephrosia*. Rotenone serves as a high-affinity noncompetitive inhibitor of complex I, thus affecting ATP generation (Cabezas et al. 2012; Gyulkhandanyan et al. 2003). Previous epidemiological studies have supported the hypothesis that prolonged exposure to pesticides such as rotenone or paraquat is a risk factor for PD (Betarbet et al. 2000; Greenamyre et al. 2003; Tanner et al. 2011). For example, a recent case–control study from the NIH, which reviewed 110 PD cases and 358 controls, observed that PD incidence was increased 2.5 times in individuals while comparing the reported use of rotenone with nonusers (Tanner et al. 2011). Another study in agricultural workers from East Texas identified a significant increased risk (OR = 10.9) of PD with the continuous use of rotenone (Dhillon et al. 2008). Similarly, different groups have demonstrated that continuous systemic administration of rotenone to rats and mice reproduces key features of PD, including selective degeneration of the nigrostriatal dopaminergic system, microglial activation, formation of cytoplasmic inclusions in neurons, movement disorders, mitochondrial membrane potential lost, and mitochondrial damage (Betarbet et al. 2000; Greenamyre et al. 2003, 2010; Hoglinger et al. 2005; Ogawa et al. 2005).

Astrocytes play an important role in the maintenance of neuronal homeostasis by regulating neuronal function and metabolism (Barreto et al. 2011; Cabezas et al. 2012; Hamby and Sofroniew 2010; Volterra and Meldolesi 2005). Astrocytes are important sources of GSH (glutathione) and growth factors such as BDNF and GDNF, and are important for neuronal protection during neurodegenerative processes including traumatic brain injury, ischemia, and PD (Avila Rodriguez et al. 2014; Barreto et al. 2011; Cabezas et al. 2012, 2014; Giffard and Ouyang 2004; Hirsch et al. 2003; Torrente et al. 2013). A previous study indicated that astrocytes co-cultured with neurons increase neuronal viability during rotenone damage (Cao et al. 2007). In this context, astrocytic protection is of great importance for neuronal survival both during normal and pathological conditions. However, there is little information about rotenone effects on astrocytes and astrocytic-like models, such as T98G cells (glioblastoma). Previous studies have shown that different rotenone doses increase glial reactivity and ROS (reactive oxygen species) generation (Swarnkar et al. 2012), suggesting the importance of astrocytic protection during ROS processes such as that in PD development.

In the present study, we assessed the role of platelet-derived growth factor isoform B (PDGF-BB) in astrocytes treated with rotenone. PDGF-BB is widely expressed in different CNS tissues including neurons and astrocytes (Andrae et al. 2008; Krupinski et al. 1997). PDGF-BB is a dimeric protein of approximately 30 kDa, which belongs to the family of the PDGF/VEGF growth factor (vascular endothelial). At the moment, there are five identified compositions or dimeric isoforms: PDGF-AA, -BB, -AB, -CC, and -DD, and these isoforms show different abilities to bind and activate both tyrosine–kinase receptors for PDGF, PDGFRα, and PDGFRβ (Andrae et al. 2008). Different isoforms of PDGF have been shown to exert a neuroprotective effect in dementia and oxidative insult in neurons against hydrogen peroxide (Peng et al. 2012; Zheng et al. 2010; Zhu et al. 2009). However, its potential neuroprotective effect in astrocytes, and its role in the regulation of rotenone-induced oxidative mechanisms have not been determined yet. In our experiments, we used human cell line T98G, as it has been broadly used in astrocytes research since they share many features with primary astrocytes and have been successfully used in our research group (Avila Rodriguez et al. 2014; de Joannon et al. 2000; Gasque et al. 1996; Stein 1979; Torrente et al. 2013).

Materials and Methods

Cell Culture

T98G cell line (human glioblastoma) was used as cell model system, where the stock was maintained under exponential growth in DMEM (Lonza) culture medium, supplemented with 10 % fetal bovine serum (FBS) and antibiotics (penicillin/streptomycin and amphotericin; Lonza) at 37 °C and in a humidified atmosphere containing 5 % CO2.

Drug Treatment

Cells were seeded in multiwell plates and allowed to grow for 24 h. Afterward, culture medium was serum deprived for 24-h prior drug treatment. Firstly, cells were incubated in DMEM without serum and treated with increasing concentrations of rotenone (Sigma-Aldrich, R8875) in 0.5 % DMSO (vehicle) for 24 and 48 h in order to obtain the optimum time and dose conditions in our experimental paradigm. In the following experiments, a concentration of 50 μM rotenone for 24 h was selected (Supplementary Fig. 1). We observed that 0.5 % DMSO did not affect cell viability; therefore, subsequent experiments were carried out in DMEM as a control. In a second experiment, the best conditions of PDGF-BB treatment were chosen (Supplementary Fig. 2). In the following experiments, 200 ng/ml PDGF-BB (Sigma, P3201) was used prior to rotenone treatment.

Cell Viability and Nuclear Fragmentation

The effect of rotenone and PDGF-BB on T98G cell proliferation or viability was tested using MTT ((3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay (Sigma, St Louis, MO, USA). Cells were seeded into 96-well plates in the DMEM culture medium containing 10 % bovine fetal serum at a seeding density of 10,000 cells per well. Afterward, cells were serum deprived for 24 h, and finally treated according to different experimental paradigms. Viability and/or proliferation were assessed at 24 and 48 h following treatments by adding MTT solution for 3 h, and the absorbance was read at 490 nm. Each assay was performed with a minimum of six replicate wells for each condition. In addition, cells were stained with propidium iodide (PI, 50 μg/ml).

To visualize nuclear morphology, cells were washed twice with 1 mM PBS after the treatments and then fixed in 4 % paraformaldehyde for 10 min. Cells were permeabilized with 1 % Triton X-100, diluted in PBS with 2.5 % serum, for 20 min at room temperature (RT), and stored at −20 °C. The cells were then stained with 2.5 mg/ml DNA dye Hoechst 33258 in PBS for 20 min at RT and analyzed by fluorescence microscopy (Olympus IX53 microscope—ex/em 340/510 nm) using a 20X objective. Viable healthy human astrocytic T98G cells nuclei were uniformly stained. The number of cells that showed nuclear fragmentation nuclei was determined in at least eight randomly selected areas (0.03 mm^2) from each experimental group. The experiment was repeated three times. Data were expressed as a percentage of nuclear fragmented cells

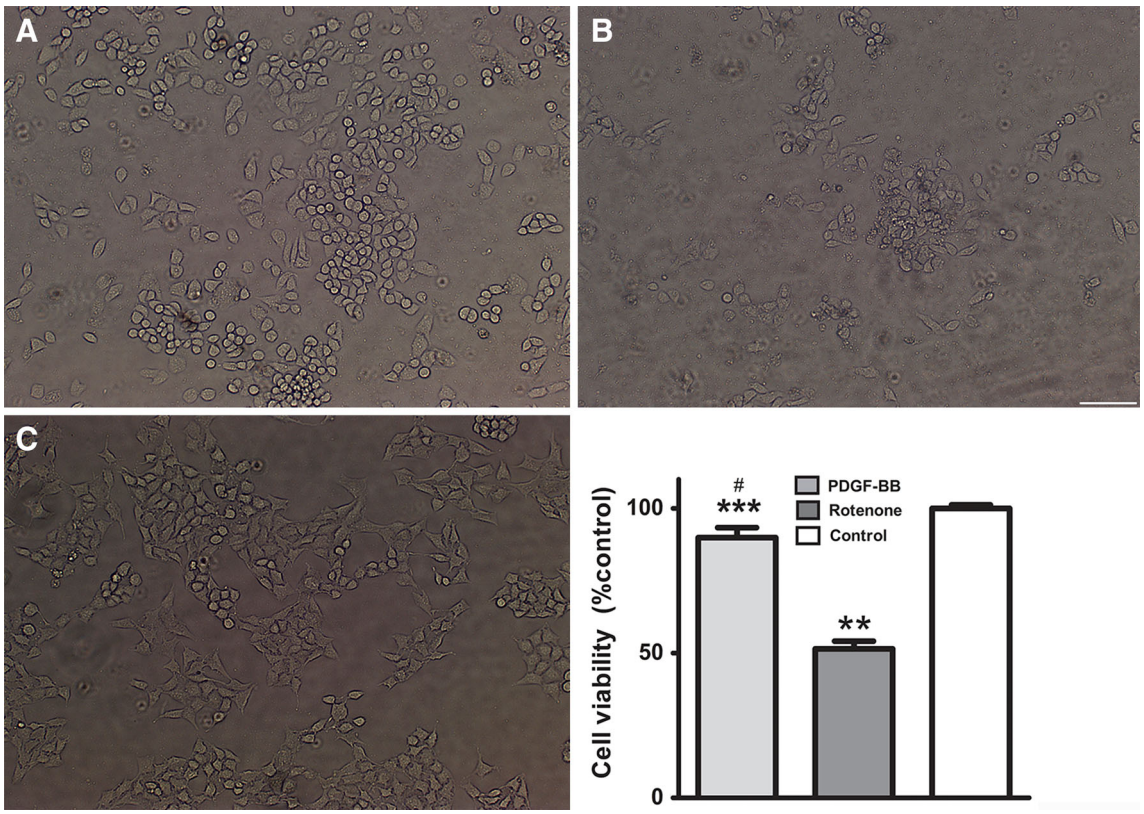

Fig. 1 Protective effects of PDGF-BB against rotenone damage. Data are expressed as percentage of control. **a** 200 ng/ml of PDGF-BB pretreatment showed a high recovery against **b** rotenone-induced effect on cell viability in comparison to **c** control. $***p < 0.0001$, PDGF-BB versus rotenone; $**p < 0.0001$, rotenone versus control; $^{\#}p < 0.05$, PDGF-BB versus control. *Scale bar* 50 μm

relative to the value in control cultures. The percentage of condensed/fragmented nuclei was determined on six replicates for each condition and normalized to controls.

Morphometric Analysis

The images were analyzed and processed using Image J software, version 1.47v. Black and white images were analyzed to assess cell area. Cells were selected randomly using a scale generated automatically (available at http://www.random.org/integers). The number of cells was estimated using the fractionator/dissector method by manually counting the number of cells in an area of 0.03 mm^2. Cell area was calculated by randomly selecting cells from the images. Data were acquired from at least six independent cultures with a minimum of 20 cells for each condition.

Image Mean Fluorescence Calculation

The calculation of Mean fluorescence of the images was assessed using ImageJ version 1.47v (Bankhead 2013) as follows: the images were opened in the software and pre-processed eliminating the background; subsequently, cells were randomly chosen via a numbered grid assigned. 20 cells were randomly selected to Mean Fluorescence calculation, and the average of eight images for each treatment were statistically analyzed. Using the Measure algorithm of ImageJ and selecting each cell manually via ROI's Management, the data were grouped and subsequently analyzed. There were no variations in the conditions of the imaged process, such as gain of the mercury lamp, time of exposure, and fluorochrome bleach. Each assay was performed with a minimum of six replicate wells for each condition. The area of each randomly selected cell was calculated using ImageJ, so the scale to calibrate ImageJ software was settled, by measuring a known distance; subsequently, the thresholds of the images were adjusted to analyze particles using the ImageJ algorithm to generate the data (range of particle size: 100–1,000 mm^2). Cells were counted in an area of 0.03 mm^2. The mean cell area was calculated for each experimental group in at least six independent cultures ($N = 6$) run in triplicate with a minimum of 20 cells being analyzed for each condition.

Determination of Reactive Oxygen Species (ROS)

Reactive oxygen species production was evaluated by fluorescence microscopy as previously described (Avila

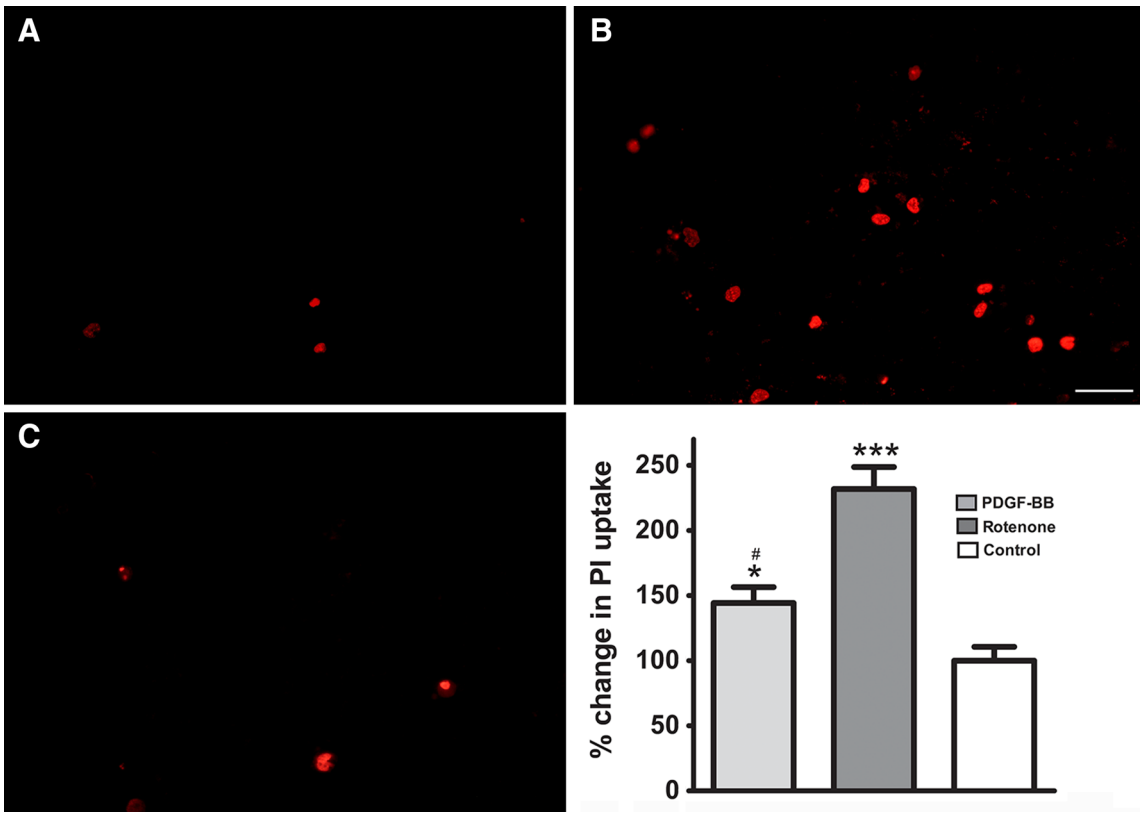

Fig. 2 PDGF-BB pretreatment protects cell viability against rotenone damage by PI staining. Data are expressed as percentage of cells expressing PI when are treated with **a** PDGF-BB pretreatment, **b** rotenone or **c** control. $^{*}p < 0.0001$, PDGF-BB versus rotenone; $^{***}p < 0.0001$, rotenone versus control; $^{\#}p < 0.05$, PDGF-BB versus control. *Scale bar* 50 μm

Rodriguez et al. 2014). In brief, cells were seeded at a density of 25,000 cells per well into 24-wellplates in DMEM culture medium containing 10 % FBS and then were treated according to each experimental paradigm after 24 h of serum deprivation. To measure the effect of PDGF-BB and rotenone on superoxide (O_2^{-}) and oxygen peroxide (H_2O_2) production, cells were treated in the dark at 37 °C for 30 min with 10 mM dihydroethidium (DHE; Sigma) or 1 mM 2′,7′-dichlorofluorescein diacetate (DCFDA), respectively. Then, cells were washed twice with PBS and photographed in an Olympus I53X fluorescence microscope. The images were processed with ImageJ software, and the mean fluorescence intensity was determined as described above for fluorescence microscopy. Each assay was performed with a minimum of six replicates for each condition. The experiment was repeated three times.

Determination of Mitochondrial Membrane Potential

Mitochondrial membrane potential was evaluated using tetramethyl rhodamine methyl ester (TMRM). TMRM is a cell-permeate, cationic fluorescent dye sequestered by active mitochondria. After 24 h of established treatments, cells were loaded in the dark with 500 nM TMRM at 37 °C for 20 min. Thereafter, cells were washed with PBS to eliminate all the unsequestered dye, and imaged as described above. As experimental control, we used the protonophoric uncoupler carbonyl cyanide *m*-chlorophenylhydrazine (CCCP; Sigma-Aldrich; 10 mM) to dissipate the membrane potential and define the baseline for the analysis of mitochondrial potential.

Determination of Mitochondrial Volume

Mitochondrial mass was evaluated using Nonyl Acridine Orange (NAO), a cell permeate, cationic fluorescent dye sequestered by active mitochondria (Oliva et al. 2011). After 24 h of treatments, cells were loaded in the dark with 5 μM NAO at 37 °C for 20 min. Thereafter, cells were washed with PBS to eliminate all the unsequestered dye. As experimental control, we used 500 μM rotenone to create a disturbance in mitochondrial membrane and increase the cardiolipin oxidation. Mitochondrial volume was further evaluated by flow cytometry in a GuavaR Easy Cyte™ (Millipore) cytometer.

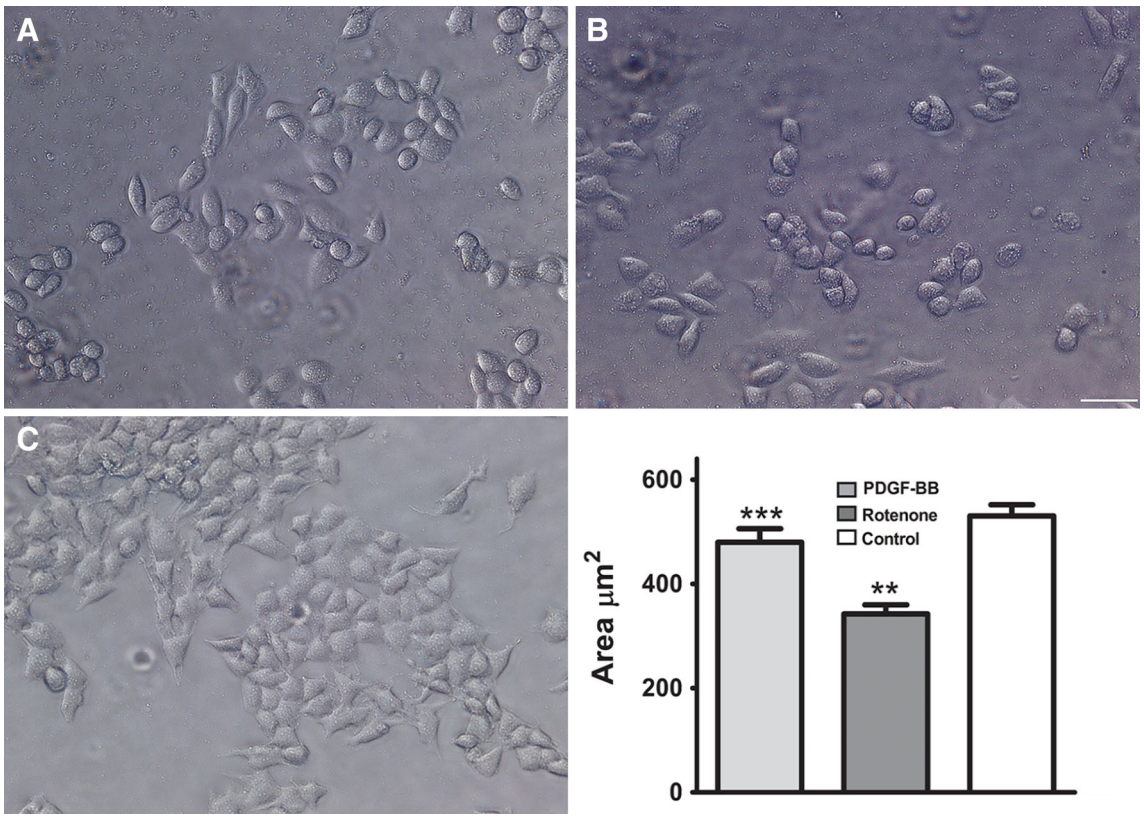

Fig. 3 Protective effects of PDGF-BB pretreatment on cellular morphology. *Bar graph* shows the changes in cell area (μm^2) for the different treatments. **a** PDGF-BB pretreatment significantly preserved the cell mean area compared with **b** rotenone-treated cells. ***$p < 0.0001$, PDGF-BB versus rotenone; **$p < 0.0001$, rotenone versus control. *Scale bar* 50 μm

Statistical Analysis

Data obtained from this study were tested for normal distribution by Kolmogorov–Smirnov test and homogeneity of variance by Levene's test. Then, data were examined by analysis of variance, followed by Dunnet's post hoc test for comparisons between controls and treatments and Tukey's post hoc test for multiple comparisons between the means of treatments and time points. Data are presented as mean ± SEM. A statistically significant difference was defined at $p < 0.05$.

Results

PDGF-BB Increased Cell Viability Against Rotenone Injury in T98G Cells

The isoflavonoid rotenone has been used in various animals and cellular models of neurodegeneration, as it increases ROS production and serves as a high-affinity noncompetitive inhibitor of complex I, thus affecting ATP generation and cell viability (Greenamyre et al. 2003, 2010). Previous studies have shown that rotenone induces apoptotic responses in mesencephalic dopaminergic cells, lymphocytes, and the neuroblastoma cell line SH-SY5Y (Avila-Gomez et al. 2010; de Oliveria et al. 2009, 2011).

Initially, we established the optimum experimental conditions of rotenone injury. Our findings indicated that the IC50 of rotenone was 50 μM at 24 h (Supplementary Fig. 1). Similarly, we found that the best protective conditions for PDGF-BB against rotenone damage were observed when the compound at 200 ng/ml was administered 24 h prior to rotenone insult (Supplementary Fig. 2). PDGF-BB pretreatment for 24 h increased the cell viability by 39 % in comparison with cells treated with rotenone alone (Fig. 1). This result was later confirmed by assessing PI uptake (50 ng/ml) in T98G cells treated with both rotenone and PDGF-BB (Fig. 2). Rotenone caused a significant increase in PI uptake when compared with cells treated with PDGF-BB (131.8 ± 16.92 vs. 44.3 ± 12.14 %; $p < 0.0001$). Based on these preliminary results, we used these parameters (rotenone and PDGF-BB concentrations and time of administration) in the next experiments.

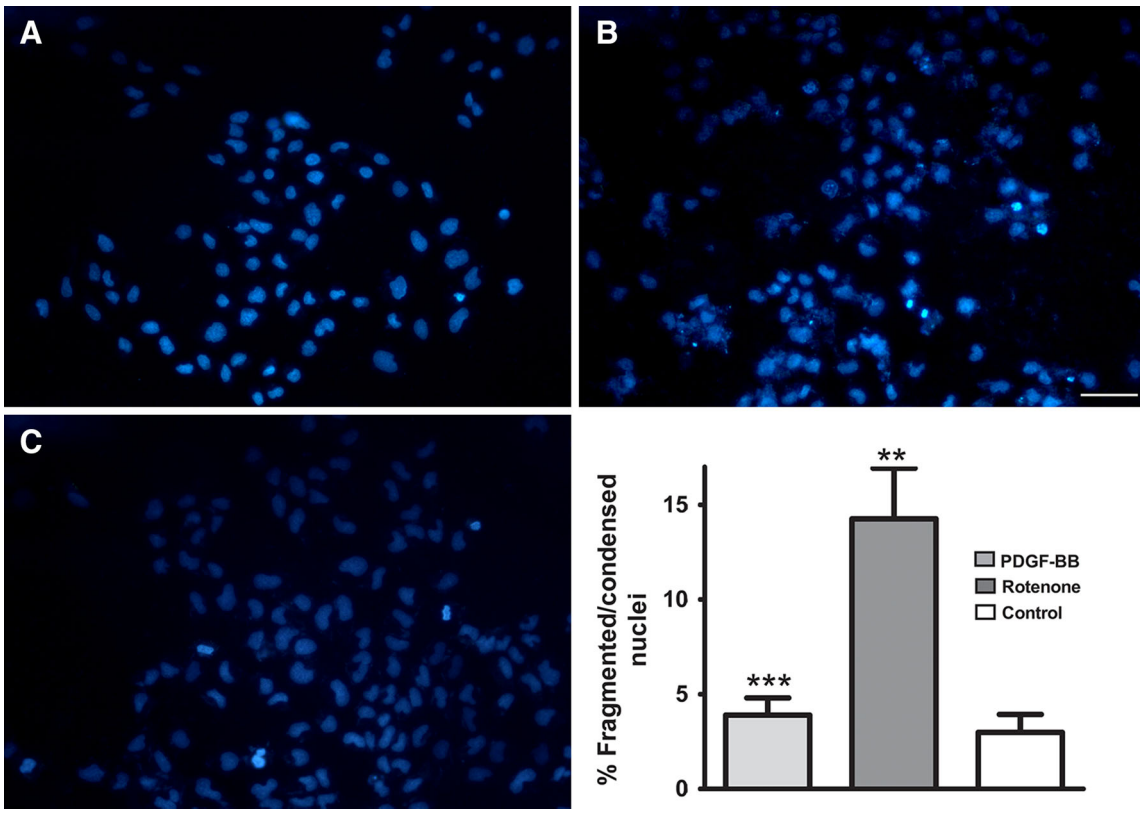

Fig. 4 PDGF-BB pretreatment decreased the number of condensed nuclei following rotenone insult. **a** PDGF-BB pretreatment significantly reduced the number of condensed and fragmented nuclei compared with **b** rotenone-treated cells. ***$p < 0.0001$, PDGF-BB versus rotenone; **$p < 0.0001$, rotenone versus control. *Scale bar* 50 μm

PDGF-BB Decreased Morphological Changes Induced by Rotenone

Previous studies have reported a positive correlation between cell viability and morphology (Avila Rodriguez et al. 2014; Xiong et al. 2006), also indicating that augmented oxidative stress may induce important morphological changes. Next we evaluated whether PDGF-BB was able to preserve cell morphology under inflammatory oxidative stress with rotenone. We observed a qualitative shrinkage of the cell body and processes when cells are exposed to rotenone (Fig. 3). Importantly, PDGF-BB-pretreated cells resulted in a preserved cellular mean area in comparison with cells exposed to rotenone only (480 ± 26.09 vs. 364.20 ± 17.47 μm^2; $p < 0.0001$). Furthermore, no significant difference was observed in cells treated with PDGF-BB in respect of controls ($p > 0.05$). These results suggested that PDGF-BB effectively preserved cell morphology against rotenone.

To further investigate the mechanisms of protection found in the previous evidences, we assessed the effect of PDGF-BB on nuclear condensation and fragmentation under rotenone treatment by Hoechst 33258 staining. Previous studies have suggested that rotenone induced the nuclear condensation and fragmentation in different cell models, this event being described as the first indication of apoptotic process in the nervous system cells (Swarnkar et al. 2012; Watabe and Nakaki 2004). Figure 4 shows that 24 h of rotenone treatment increased the nuclear condensation and fragmentation in T98G cells compared with control ($p < 0.0001$). In contrast, PDGF-BB pretreatment significantly attenuated the number of fragmented nuclei compared with rotenone only (3.89 vs. 14.26 %, respectively; $p < 0.0001$). No significant difference was detected between PDGF-BB and control ($p > 0.05$).

PDGF-BB Reduced ROS Levels in Cells Stimulated with Rotenone

One of the main damage exerted by rotenone is an increase in the production of ROS, especially superoxide anions and peroxides (Dick 2006). Rotenone induced a significant increase of 46.40 % on ROS production measured by DHE ($p = 0.0013$). On the other hand, PDGF-BB pretreatment maintained superoxide production in a level similar to that observed in control (98.72 vs. 100 %, respectively; Fig. 5).

To further assess the protective mechanism of PDGF-BB, we determined the changes exerted by this growth factor on hydrogen peroxides by measuring DCFH-DA fluorescence. Rotenone induced a significant increase on

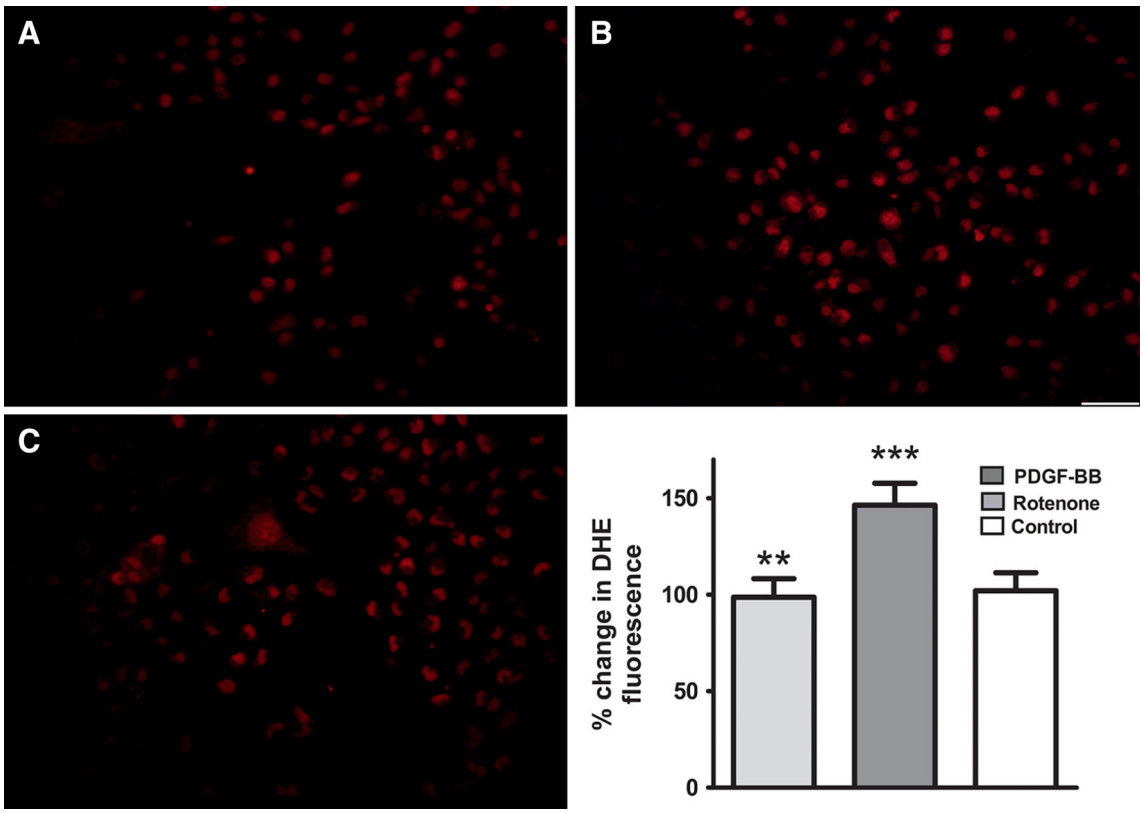

Fig. 5 PDGF-BB pretreatment reduced ROS production against rotenone. The *bar graphs* show the mean values of DHE fluorescence of cells treated with **a** PDGF-BB, **b** rotenone **c** and control. $^{**}p < 0.0013$, PDGF-BB versus rotenone; $^{***}p < 0.0014$, rotenone versus control. *Scale bar* 50 μm

peroxide production of 75 % ($p < 0.0001$) compared with control. PDGF-BB pretreatment, however, reduced peroxide fluorescence ($p < 0.0001$, Fig. 6), further corroborating the protective effect of PDGF-BB on ROS production.

PDGF-BB Partially Reversed the Mitochondrial Membrane Potential $\Delta\psi_m$ Decrease in Rotenone-Insulted Cells

The mitochondrial membrane potential $\Delta\psi_m$ was evaluated using TMRM staining, a technique that let us to assess the proportion of cells with loss of the $\Delta\psi_m$. Figure 7 shows that after 24-h rotenone treatment, T98G cells had a drastic drop on the $\Delta\psi_m$ compared with control (42.57 ± 4.02 vs. 100 %, respectively; $p < 0.0001$). PDGF-BB pretreatment partially preserved $\Delta\psi_m$ (62.00 ± 4.80 %, $p < 0.05$), suggesting an effect of PDGF-BB on mitochondrial potential membrane protection.

PDGF-BB Reduced Rotenone-Damaging Effects on Mitochondrial Volume

Finally, we aimed to evaluate the mitochondrial volume using NAO, as this is a widely used agent for the assessment of changes in mitochondrial lipid peroxidation during apoptotic processes, and could provide additional information regarding the effect of PDGF-BB pretreatment on mitochondrial preservation (Oliva et al. 2011). After 24-h rotenone insult, we observed a great reduction on NAO fluorescence compared with PDGF-BB (61.73 ± 3.89 vs. 31.10 ± 5.84 %, $p < 0.0001$; Fig. 8). This effect was later confirmed by flow cytometry for NAO (Fig. 9). This result provides further confirmation of PDGF-BB effect on mitochondrial protection.

Discussion

The importance of growth factors in neuroprotection has been widely assessed in previous research mainly in neuronal models, as they are important for CNS homeostasis, differentiation, and survival (Falk et al. 2009; Lee et al. 1997; Mattson et al. 1997; Safi et al. 2012). Growth factors such as BDNF and bFGF have shown to protect neurons against excitotoxicity through the increased expression of antioxidant enzymes such as Mn-SOD and glutathione reductase (Lee et al. 1997; Mattson et al. 1997). Furthermore, previous studies have demonstrated that different

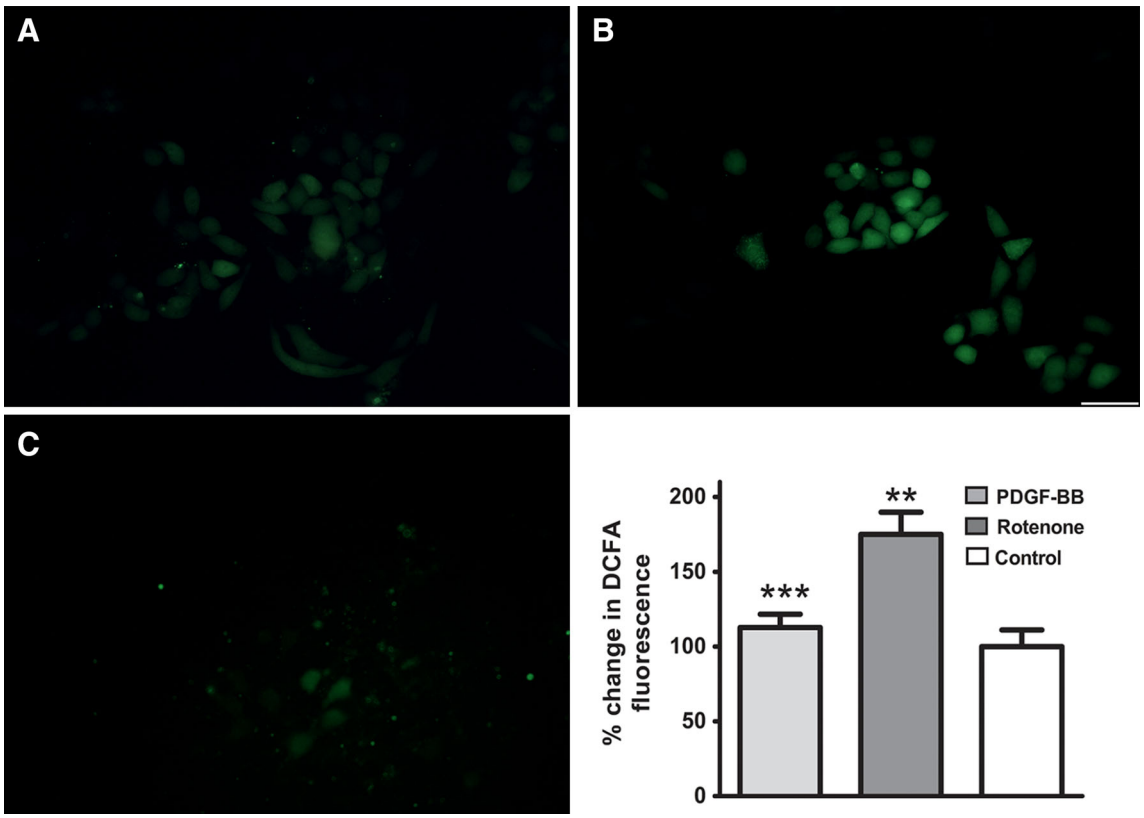

Fig. 6 PDGF-BB pretreatment attenuated peroxide production after rotenone treatment. *Bar graph* shows the values of DCFA fluorescence when cells are exposed to **a** PDGF-BB, **b** rotenone, and **c** controls. ***$p < 0.0001$, PDGF-BB versus rotenone; **$p < 0.0001$, rotenone versus control. *Scale bar* 50 μm

PDGF isoforms can protect neuronal cells against different insults such as H_2O_2, VIH TAT toxin, and 6-OHDA (Tang et al. 2010; Zheng et al. 2010; Zhu et al. 2009). Despite the vast data on neuronal protection induced by these compounds, very few studies have focused on the protective mechanisms of growth factors in non-neuronal cells such as astrocytes. Astrocytes are important for the physiological regulation of neurons through the release of growth factors like NGF and bFGF, antioxidant molecules such as glutathione, and clearance of toxic α-synuclein, and glutamate. However, these astrocytic functions become greatly affected during the development of neurodegenerative diseases, thus resulting in an increased neuronal death (Barreto et al. 2011; Rappold and Tieu 2010), suggesting the importance of astrocyte protection during neurodegeneration. In the present study, we used the astrocyte-like model T98G glioblastoma cells, and assessed some effects of PDGF-BB on mitochondrial functions following rotenone treatment. This is the first study to assess the role of PDGF-BB on astrocytes-like cells under rotenone-induced oxidative stress. Our results indicated that PDGF-BB decreased the neurotoxic effects of rotenone on T98G cell death, nuclear fragmentation, free radicals' production, mitochondrial membrane's potential, and morphology and suggest that glial cells may participate in the neuroprotective actions of PDGF-BB. This important effect of PDGF-BB in astrocytic protection is in agreement with the neuroprotective role of trophic factors, such as BDNF and bFGF, thus demonstrating the importance of growth factors in non-neuronal cellular protection (Lee et al. 1997; Mattson et al. 1997).

Rotenone has been widely used in neurotoxic models as it causes molecular and cellular processes similar to those observed in neurodegenerative disease like PD (Cabezas et al. 2012; de Oliveria et al. 2009, 2011; Greenamyre et al. 2003; Valverde et al. 2008). Rotenone has a direct effect on mitochondrial functions, including an interference with the electron transport chain, loss of mitochondrial membrane's potential, and ATP generation (Greenamyre et al. 2003; Sarafian et al. 2010; Simpkins et al. 2010). These cellular processes in turn cause the release of cytochrome C from the inner mitochondrial membrane, thus activating apoptotic effectors such as Bid, Bax, caspase 3, and 9 in neurons (Gyulkhandanyan et al. 2003; Swarnkar et al. 2012; Tiwari et al. 2011; Wang et al. 2011). Similarly, our experiments showed that 50 μM rotenone drastically affected cell

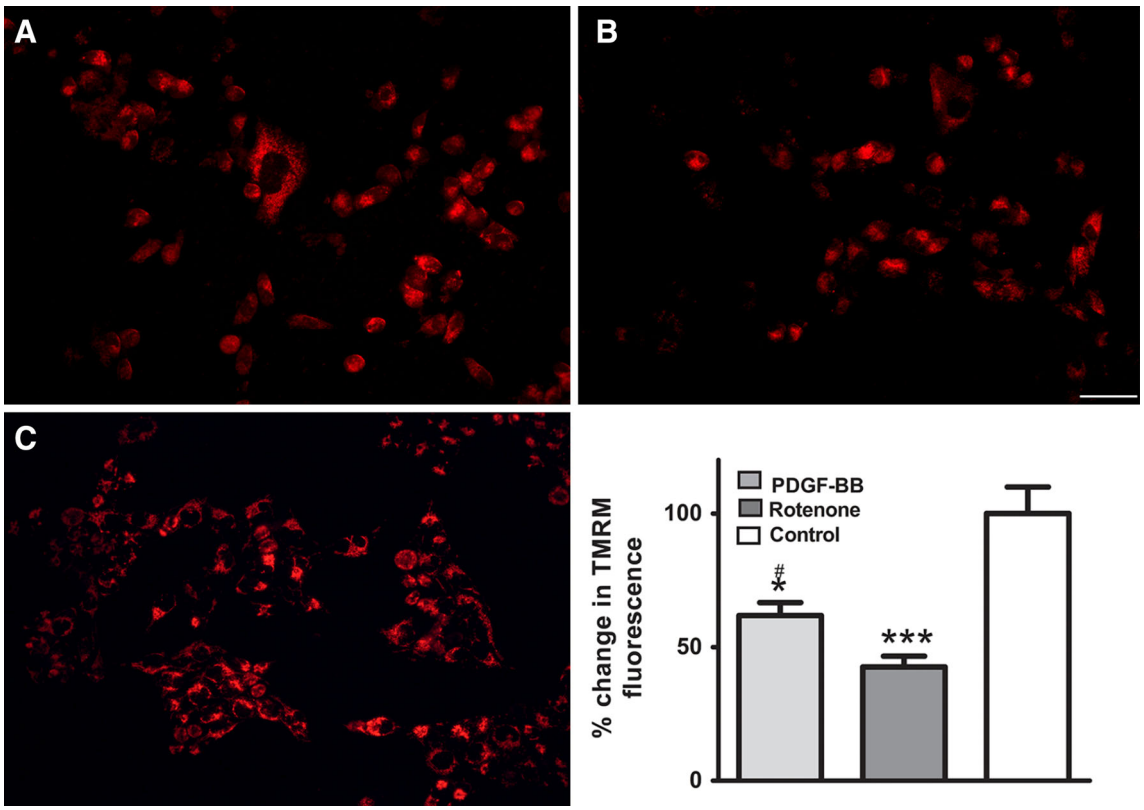

Fig. 7 PDGF-BB pretreatment partially preserved mitochondrial membrane potential in cells stimulated with rotenone. *Bar graph* shows the values of TMRM fluorescence of cells treated with **a** PDGF-BB, **b** rotenone, and **c** controls. $^{*}p < 0.05$, PDGF-BB versus rotenone; $^{***}p < 0.0001$, rotenone versus control. $^{\#}p < 0.0001$, PDGF-BB versus control. *Scale bar* 50 μm

viability and PI uptake in T98G cells, thus confirming previous reports.

Our results suggest that PDGF-BB diminished the damaging effects of rotenone by preserving several functions in the cell, including viability, ROS production, and maintenance of mitochondrial functions. Importantly, PDGF-BB protective responses were more effective during pretreatment conditions. Previous studies in neuronal models have also shown that 24-h pretreatment with PDGF-BB exerts a significative protection against different insults such as hydrogen peroxide, glucose deprivation, and cytotoxic death in cultured neurons (Cheng and Mattson 1995; Tseng and Dichter 2005; Zheng et al. 2010). PDGF-BB preconditioning was shown to induce PDGFRB phosphorilation, followed by an activation of downstream effectors like PI3K/AKT and MAPK, an increase in antioxidant enzymes like catalase and gluthatione peroxidase and increased expression of antiapoptotic genes like Bcl-xL and Bax (Cheng and Mattson 1995; Zheng et al. 2010). Our results have also shown that PDGF-BB protected against the morphological shrinking produced by rotenone insult, which may be strongly related with the observed changes in cell viability (Xiong et al. 2006). In this aspect, PDGF-BB has been involved in cellular morphological regulation through the activation of actine fibers and GTP protein RhoA both in fibroblasts and endothelial cells (Ruusala et al. 1998), suggesting an effect of PDGF-BB on the maintenance of cellular morphology under rotenone insult. It is possible that PDGF-BB effects on T98G cells may signal through activation of PDGFRβ, as previous studies indicated that this cell line has a higher expression of PDGFRβ compared with PDGFRα isoform (Lokker et al. 2002; Potapova et al. 1996).

Mitochondria are critical organelles for cell survival and normal development, as they provide energy to the cell; are essential in ROS production; and are key regulators of apoptotic death (Simpkins et al. 2010). Moreover, the maintenance of mitochondrial properties in both neurons and astrocytes is of central importance during brain injury and neurodegeneration (Barreto et al. 2011; Greenamyre et al. 2003, 2010). The impairment of astrocytic mitochondrial functions, such as a decrease in ATP production or mitochondrial potential, has been correlated to increased neuronal damage in co-cultured models (Barreto et al. 2011; Volobouéva et al. 2007), thus demonstrating the importance of astrocytic mitochondrial maintenance during neuroprotection.

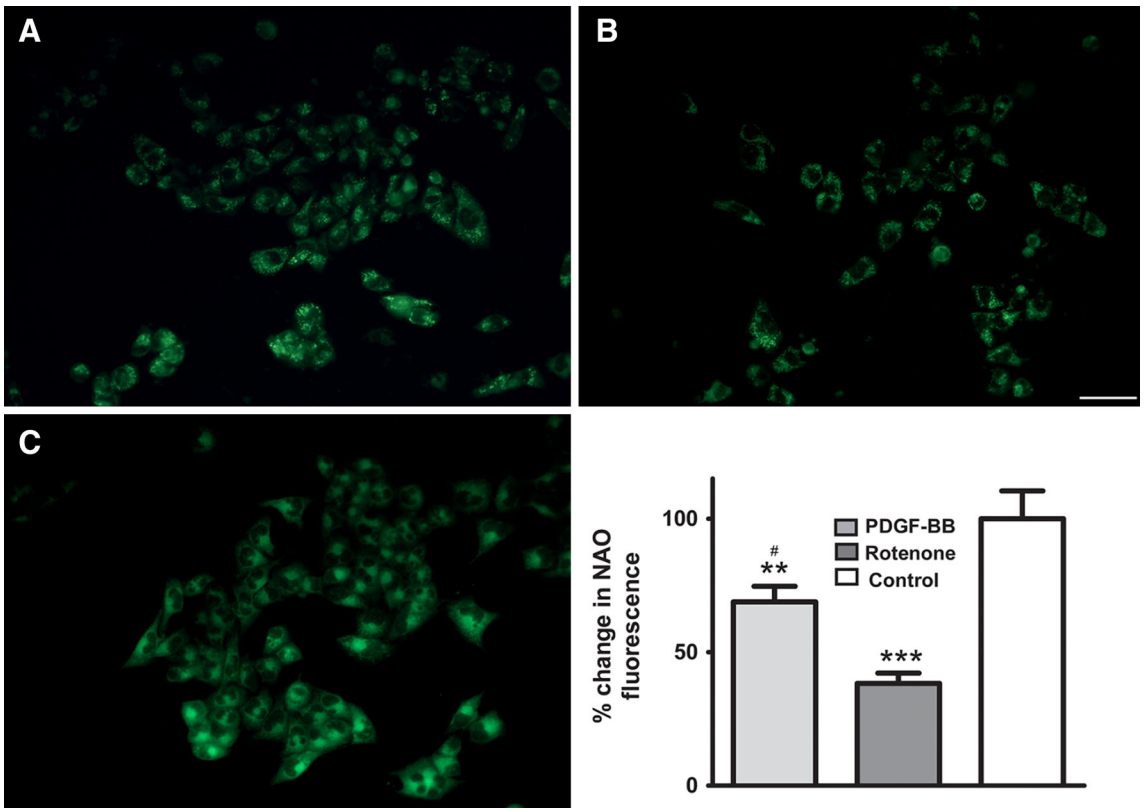

Fig. 8 PDGF-BB pretreatment preserved mitochondrial volume against rotenone. The *bar graph* shows the values of NAO fluorescence in cells pretreated with **a** PDGF-BB pretreatment followed by **b** rotenone stimuli. $^{**}p < 0.05$, PDGF-BB versus rotenone; $^{***}p < 0.0001$, rotenone versus control; $^{\#}p < 0.05$, PDGF-BB versus control. *Scale bar* 50 µm

Few studies have shown the importance of PDGF-BB and other growth factors in mitochondrial regulation. The present study showed that PDGF-BB pretreatment reduced both superoxide and peroxide production induced by rotenone in T98G cells as quantified by DHE and DCFA-DA. Similarly, it has been shown that PDGF-BB suppresses peroxide accumulation in neurons and rescues cell viability against H_2O_2, possibly through PI3K and MAPK activation followed by an increase in catalase, glutathione peroxidase, and GSH content (Cheng and Mattson 1995; Iantomasi et al. 1999; Zheng et al. 2010). The maintenance of $\Delta\psi_m$ is also essential for cell survival and ATP production, and its depolarization has been associated with damaging processes such as PD and metabolic injuries like glucose deprivation, similar to what is observed during rotenone insult (Avila Rodriguez et al. 2014; Barreto et al. 2011; Greenamyre et al. 2010; Perier and Vila 2012). Our findings also indicated that PDGF-BB protected the $\Delta\psi_m$ depolarization against rotenone in our astrocytic-like model. Similarly, Avila-Gomez et al. (2010) demonstrated that IGF-I pretreatment for 24 h protected the lymphocytes against rotenone by activation of PI-3K/Akt/β-catenin, downregulation of p53 regulation, and maintenance of mitochondrial potential, suggesting a possible interaction with the PI3K/AKT pathway and protection of mitochondrial function. It is possible that a similar mechanism may be playing a role in the observed protection of $\Delta\psi_m$ by PDGF-BB in our findings. However, further experiments are required to address this point. Similarly, our results by NAO demonstrated that PDGF-BB protected T98G cells against mitochondrial mass loss induced by rotenone. In this aspect, a previous study by Gosslau et al. (2001) have reported that PDGF-AB increased mitochondrial volume and surface area of mitochondrial cristae in injured fibroblasts determined by electron microscopy, suggesting an effect of PDGF-AB on mitochondrial preservation. Interestingly, various studies have reported that alterations in mitochondrial morphology and bioenergetic balance are associated to diseases such as type 2 diabetes, Huntington, and Parkinson diseases, demonstrating the importance of mitochondrial preservation in the development of neuropathologies (Arduino et al. 2011; Chaturvedi and Beal 2013; Perier and Vila 2012).

In conclusion, our results demonstrated the protective effects of PDGF-BB in the astrocytic-like model against rotenone-induced oxidative stress. PDGF-BB was also shown to improve mitochondrial protection, thus indicating that PDGF-BB signaling pathways may have an effect on

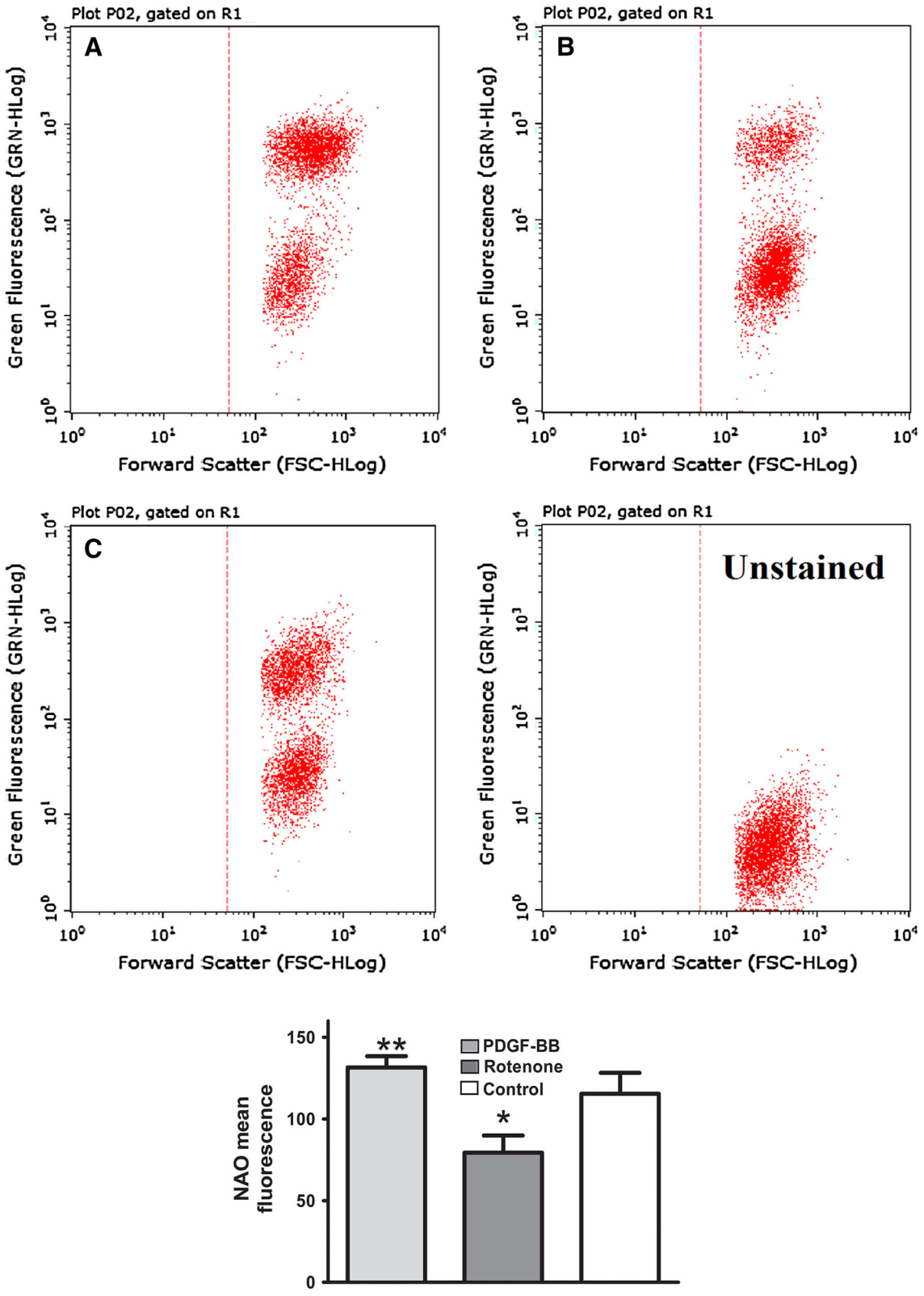

Fig. 9 Representative flow cytometry plots of NAO fluorescence. Cells were exposed to **a** PDGF-BB pretreatment, **b** rotenone, and **c** as control. The *bar graph* shows the value of NAO fluorescence in arbitrary units. $^{**}p < 0.01$, PDGF-BB versus rotenone; $^{*}p < 0.05$, rotenone versus control

mitochondrial preservation. However, further experiments are needed to confirm this hypothesis using different models such as astrocyte primary cultures.

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Conflict of interest The authors declare no conflict of interest.

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