Mitochondrial ATP-sensitive potassium channels enhance angiotensin-induced oxidative damage and dopaminergic neuron degeneration. Relevance for aging-associated susceptibility to Parkinson's disease

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Abstract Recent studies have shown that renin–angiotensin system overactivation is involved in the aging process in several tissues as well as in longevity and aging-related degenerative diseases by increasing oxidative damage and inflammation. We have recently shown that angiotensin II enhances dopaminergic degeneration by increasing levels of reactive oxygen species and neuroinflammation, and that there is an aging-related increase in angiotensin II activity in the substantia nigra in rats, which may constitute a major factor in the increased risk of Parkinson's disease with aging. The mechanisms involved in the above mentioned effects and particularly a potential angiotensin–mitochondria interaction have not been clarified. The present study revealed that activation of mitochondrial ATP-sensitive potassium channels

[mitoK(ATP)] may play a major role in the angiotensin II-induced effects on aging and neurodegeneration. Inhibition of mitoK(ATP) channels with 5-hydroxydecanoic acid inhibited the increase in dopaminergic cell death induced by angiotensin II, as well as the increase in superoxide/superoxide-derived reactive oxygen species levels and the angiotensin II-induced decrease in the mitochondrial inner membrane potential in cultured dopaminergic neurons. The present study provides data for considering brain renin–angiotensin system and mitoK(ATP) channels as potential targets for protective therapy in aging-associated diseases such as Parkinson's disease.

Keywords Aging · Neurodegeneration · Neuroinflammation · Renin–angiotensin system · Oxidative stress · Parkinson's disease

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Introduction

Increased activity of local renin–angiotensin system, acting via angiotensin II type 1 receptors (AT1), is thought to be involved cellular senescence and age-related degenerative changes in several tissues (Basso et al. 2005; Min et al. 2009; Mukai et al. 2002). In accordance with this, recent studies with angiotensin II type 1 receptor deficient mice indicate that disruption of this receptor promotes longevity through

attenuation of oxidative stress and additional mechanisms (Benigni et al. 2009, 2010; Mattson and Maudsley 2009), and completely protects against the age-related progression of atherosclerosis (Umemoto 2008). It is known that angiotensin II, acting via type 1 receptors, is one of the most important known inducers of inflammation and oxidative stress in several tissues (Mattson and Maudsley 2009; Min et al. 2009; Ruiz-Ortega et al. 2001). Normal aging has been associated with a pro-inflammatory, pro-oxidant state that may favor an exaggerated response to injury and degenerative diseases (Choi et al. 2010; Csizsar et al. 2003; Ungvari et al. 2004). In accordance with this, several recent studies have suggested the potential of inhibition of angiotensin for treatment of age-associated diseases and longevity (Benigni et al. 2009, 2010; Mattson and Maudsley 2009; Nishiyama et al. 2009).

Advancing age itself is one of the most significant risk factors for the development of neurodegenerative diseases such as Parkinson's disease (PD; Collier et al. 2007; Cruz-Muros et al. 2009; Deng et al. 2006; McCormac et al. 2004). Brain possesses a local renin–angiotensin system (Mckinley et al. 2003; Saavedra 2005), and we have recently observed an aging-related increase in angiotensin activity in the nigra that leads to pro-inflammatory and pro-oxidative changes, which may constitute a major factor in the increased risk of PD with aging (Villar-Cheda et al. 2010b). It is known that neuroinflammation, oxidative stress, and microglial NADPH oxidase activation play a major role in dopaminergic neuron degeneration and PD (Rodriguez-Pallares et al. 2007; Wu et al. 2002, 2003). Furthermore, we have shown that angiotensin II, via type 1 receptors, enhances the dopaminergic degeneration process triggered by low/sublethal doses of dopaminergic neurotoxins by amplifying intra-neuronal levels of reactive oxygen species (ROS) and the inflammatory response via activation of microglial NADPH oxidase (Joglar et al. 2009; Rey et al. 2007; Rodriguez-Pallares et al. 2008).

The mechanisms involved in the above mentioned effects of increased angiotensin activity and particularly a potential angiotensin–mitochondria interaction have not been clarified. It is well known that angiotensin II acts via type 1 receptors to release high levels of ROS mainly by activation of the NADPH oxidase (Qin et al. 2004; Seshiah et al. 2002; Touyz et al. 2002), which was also observed in

the nigrostriatal system (Joglar et al. 2009; Rey et al. 2007; Rodriguez-Pallares et al. 2008). However, recent studies suggest that angiotensin II may stimulate not only cytosolic but also mitochondrial-ROS generation (de Cavanagh et al. 2007; Zhang et al. 2007). In addition, a number of studies support a critical role for mitochondrial ATP-sensitive potassium channels [mito(KATP)] in modulating intracellular ROS (Mattson and Liu 2003; Costa and Garlid 2008). A cross-talk signaling between the NADPH oxidase and the mitoK(ATP) channels has been shown (Daiber 2010; Kimura et al. 2005a; Zhang et al. 2007). This includes not only that NADPH oxidase modulates mitochondrial superoxide (Doughan et al. 2008; Kimura et al. 2005a) but also that mitochondrial superoxide stimulates extramitochondrial NADPH oxidase activity in a feed-forward fashion (Dikalova et al. 2010; Wosniak et al. 2009). In the present study, we used primary cultures of ventral mesencephalon to investigate the possibility that mitoK(ATP) channels are involved in the enhancing effect of angiotensin II on dopaminergic neuron degeneration and potentially the increased risk and progression of PD with aging.

Methods

Primary mesencephalic cultures

Ventral mesencephalic tissue was dissected from rat embryos of 14 days of gestation (E14). All experiments were carried out in accordance with the “Principles of laboratory animal care” (NIH publication No. 86-23, revised 1985) and approved by the corresponding committee at the University of Santiago de Compostela. The tissue was incubated in 0.1% trypsin (Sigma, St. Louis, MO, USA), 0.05% DNase (Sigma), and DMEM (Invitrogen, Paisley, Scotland, UK) for 20 min at 37°C, and then washed in DNase/DMEM and mechanically dissociated. The resulting cell suspension was centrifuged at 50×g for 5 min, the supernatant was carefully removed, and the pellet resuspended in 0.05% DNase/DMEM to the final volume required. The number of viable cells in the suspension was estimated with acridine orange/ethidium bromide. Cells were plated onto 35-mm culture dishes (Falcon, Becton Dickinson, Franklin Lakes, NJ, USA) previously coated with poly-L-lysine (100 µg/ml; Sigma) and laminin (4 µg/ml; Sigma). The cells

were seeded at a density of 1.5×10^5 cells/cm² and maintained under control conditions [DMEM/HAMS F12/ (1:1) containing 10% fetal bovine serum (FBS; Biochrom KG, Berlin, Germany)]. The cell cultures were maintained in a humidified CO₂ incubator (5% CO₂; 37°C) for 8 days in vitro (DIV; see below); the entire medium was removed on day 2 and replaced with fresh culture medium.

To obtain neuron-enriched cultures, cytosine- β -D-arabino-furanoside (Ara C; 1 μ M; Sigma) was added 48 h after the cells were seeded. The cultures were then treated with the dopaminergic neurotoxins 6-OHDA (6-hydroxydopamine) or MPP⁺ (1-methyl-4-phenylpyridinium), or 6-OHDA or MPP⁺ and 5-HD (5-hydroxydecanoic acid, see below). This method can enrich neurons to >85% purity (Michel et al. 1997; Gao et al. 2003).

Treatment of cultures and experimental design

Cultures were exposed on 7 DIV to 6-OHDA alone for 24 h (10 or 30 μ M, in 0.02% saline ascorbate; Sigma), or on 4 DIV to MPP⁺ alone for 4 days (0.25 μ M; Sigma), or 6-OHDA or MPP⁺ and angiotensin II (100 nM; Sigma), or 6-OHDA + angiotensin II + the angiotensin II type 1 receptor antagonist ZD 7155 (1 μ M), or 6-OHDA or MPP⁺ and 5-HD [a specific blocker of mitoK(ATP) channels, Sigma, 10 μ M], or 6-OHDA or MPP⁺ + angiotensin II + 5-HD, or angiotensin II alone, or ZD 7155 alone, or 5-HD alone, in order to study the effect on survival of dopaminergic neurons. 5-HD is the most widely used specific inhibitor of mitoK(ATP) channels, and several studies have shown that 5-HD (10 μ M) blocks the mitoK(ATP) channels without any effect on cell membrane K(ATP) channels (Costa and Garlid 2008; Garlid et al. 1997; McCullough et al. 1991; Zhang et al. 2007). Several doses of 5-HD were tested in the present and preliminary experiments (10, 100, 500 μ M; Rodriguez-Pallares et al. 2009), and the lowest dose that proved effective (10 μ M) was used in most of the experiments to prevent potential non-specific side effects of high doses of 5-HD (Wu et al. 2006). In addition, some cultures were treated with a second K(ATP) channel blocker (glibenclamide, 10 μ M, Sigma) to confirm the involvement of K(ATP) channels in AII-induced cell death.

The cells were then washed and processed for immunolabeling as detailed below. Some control

cultures and cultures subjected to the above mentioned treatments were also treated with MitoTracker Orange (CMTMR; chloromethyl-tetramethylrosamine methyl ester; Molecular Probes, Eugene, OR, USA) or MitoTracker Green FM (MTGFM; 2-[3-[5,6-dichloro-1,3-bis[[4-(chloromethyl)phenyl]methyl]-1,3-dihydro-2H-benzimidazol-2-ylidene]-1-propen-1-yl]-3-methyl-benzoxazoliumchloride; Molecular Probes; 50 nM) or dihydroethidium (DHE) for 3 h to estimate the mitochondrial inner membrane potential ($\Delta\psi_M$), the mitochondrial mass or levels of superoxide/superoxide-derived ROS, respectively, in dopaminergic neurons or microglial cells, as detailed below. Furthermore, the hyperpolarizing effect of 5-HD was confirmed in live cell cultures with JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide; Invitrogen), as described below. Finally, some cultures were pre-treated with the mitochondrial uncoupler CCCP (carbonyl cyanide *m*-chlorophenylhydrazone; Sigma, 10 μ M) to confirm that the increase in CMTMR or JC-1 retention is not due to non-specific effects and is dependent on $\Delta\psi_M$. The protonophore CCCP is often used to dissipate the membrane potential and to define the baseline for the analysis of mitochondrial membrane potential with fluorescent dyes (Gottlieb et al. 2003; Brown et al. 1996).

Immunohistochemistry and double-fluorescence labeling

Cultures were fixed with 4% paraformaldehyde in Dulbecco's phosphate buffered saline (DPBS; pH 7.4) for 20 min, and then incubated at 4°C with a mouse monoclonal anti-tyrosine hydroxylase (TH; Sigma; 1:30,000) as dopaminergic marker. Neuron-enriched cultures were evaluated by immunocytochemical staining with a mouse monoclonal anti-NeuN (Chemicon; Temecula, CA, USA; 1:2,000) as a neuronal marker, a mouse monoclonal anti-glial fibrillary acidic protein (GFAP; Chemicon; 1:1,000) as an astrocyte marker, and a mouse monoclonal anti-CD11b (anti-complement receptor-3, clone MRC OX42; Serotec, Kidlington, Oxford, UK; 1:1,000) as a marker of resting and reactive microglial cells/macrophages. Cultures were then washed and incubated for 1 h with biotinylated horse anti-mouse antibody (Vector; Burlingame, CA, USA) diluted 1:500, and then incubated for 90 min with avidin–biotin–peroxidase

complex (ABC, Vector, 1:500). Finally, the labeling was revealed with 0.04% hydrogen peroxide and 0.05% 3,3'-diaminobenzidine (Sigma) as chromogen (see Rodriguez-Pallares et al. 2008 for details).

Cultures grown on glass coverslips were processed for double-fluorescence labeling for DHE or CMTMR or MTGFM and TH or OX42. For TH or OX42, cultures were incubated overnight at 4°C with primary anti-TH (1:30,000) or anti-OX42 (Serotec, 1:1,000) antibodies. The cultures were rinsed with DPBS, then incubated for 150 min with the secondary antibodies [goat anti-mouse (Chemicon; 1:100) conjugated with fluorescein isothiocyanate (FITC) for TH, or biotinylated horse anti-mouse for OX42 (Vector; 1:500)]. OX42 labeling was visualized by incubation of the cultures with streptavidin conjugated with FITC (Sigma; 1:200) for 30 min. Co-localization of markers (see below for DHE, MTGFM, and CMTMR) was confirmed by confocal laser microscopy (TCS-SP2; Leica, Heidelberg, Germany) and use of a sequential scan method to avoid any possible overlap. In all experiments, the control cultures, in which the primary antibody was omitted, were immunonegative for these markers.

Estimation of $\Delta\psi_M$ with CMTMR (MitoTracker Orange) or JC-1, and mitochondrial mass with MTGFM (MitoTracker Green FM)

CMTMR (MitoTracker Orange TM) enters mitochondria of living cells in proportion to the negative charge difference between the cytoplasm and the mitochondrial matrix, and therefore provides an estimation of $\Delta\psi_M$. Lipophilic cations accumulate in the mitochondrial matrix, driven by the $\Delta\psi_M$ in accordance with the Nernst equation, which predicts that every 61.5-mV increase in membrane potential causes a ten-fold increase in accumulation of the membrane-permeant cation. CMTMR binds irreversibly to mitochondrial matrix thiols and can be fixed for immunocytochemical localization of proteins in the same cells that have previously been exposed to CMTMR. As a result of the thiol binding, CMTMR fluorescence represents the highest level of negativity difference in the mitochondria during exposure to the dye before fixation (Sugrue et al. 1999; Wadia et al. 1998). MitoTracker Green FM (MTGFM) is a mitochondrion-selective probe that becomes fluorescent

in the lipid environment of mitochondria. MTGFM contains a thiol-reactive chloromethyl moiety, resulting in stable peptide and protein conjugates after accumulation in mitochondria and, unlike CMTMR, uptake of this probe is less dependent on $\Delta\psi_M$, thus allowing estimation of mitochondrial mass in both live and fixed cells (Metivier et al. 1998; Poot et al. 1996).

The $\Delta\psi_M$ or the mitochondrial mass in fixed cells grown in glass coverslips was estimated as follows (Sugrue et al. 1999; Wadia et al. 1998; Buckman et al. 2001). Three hours after exposure to treatments, the medium in each well was supplemented with 50 nM CMTMR or 50 nM MTGFM and incubated at 37°C 5% CO₂ for 15 min or 30 min, respectively. The media was removed and the cells were rinsed with cold DPBS, followed by immediate fixation with 4% paraformaldehyde for 10 min. After fixation, the cells were washed briefly in DPBS and incubated for 20 min with a mouse monoclonal anti-TH (Sigma, 1:6,000) containing normal goat serum and 0.3% Triton X-100 diluted in DPBS-BSA. Cultures were then washed and incubated for 15 min with goat anti-mouse secondary antibody conjugated with FITC for CMTMR or with cyanine 3.18 (Cy3) for MTGFM. The coverslips were mounted onto microscope slides with 1,4-diazabicyclo [2.2.2] octane (DABCO; Sigma).

JC-1 is a cationic carbocyanine dye that accumulates in mitochondria. The dye exists as a monomer at low concentrations and yields green fluorescence. At higher concentrations, the dye forms J-aggregates that exhibit a broad excitation spectrum and an emission maximum at 590 nm. The JC-1 dye is only uptaken up by viable cells and it exhibits a spectral shift from green to red in healthy mitochondria with polarized membranes. The JC-1 assay was performed to estimate the $\Delta\psi_M$ in living cells, according to Zhang et al. (2006) as follows. Cells were plated in 96-well plates at 10⁵ cells per well. After treatment with 5-HD, JC-1 (in DMSO) was added to each well to a final concentration of 3 μ M and incubated for 30 min at 37°C, with 5% CO₂, in the dark. The medium was removed and the cells washed twice with DPBS. Fluorescence intensity was measured in a multifunctional microplate reader (TECAN Infinite 200, Austria) at excitation 485 nm, emission 525 and 595 nm, for detection of the green and red substrate, respectively. The fluorescence signal represents the average signal of the total cell population. The mitochondrial membrane potential is shown as the ratio between the

fluorescence of aggregate (red) and monomer (green) forms of JC-1. This ratio is dependent only on the mitochondrial membrane potential, and not on the number of cells, mitochondrial size, shape, or density.

Detection of intracellular superoxide anion/superoxide-derived ROS with dihydroethidium (DHE): estimation of changes in fluorescence intensity

Treatment with DHE is one of the most frequently used methods for the detection of intracellular superoxide. For years, it was assumed that intracellular DHE is oxidized to ethidium, which bound DNA to form a red fluorescent product. However, recent studies have shown that the fluorescence derived from the reaction between superoxide and DHE is due to accumulation of 2-hydroxyethidium and detects superoxide in the cytoplasm (Dikalova et al. 2010; Fink et al. 2004; Zhao et al. 2005). Cultures grown on glass coverslips were incubated with a fresh working solution containing 5 μ M DHE (Sigma) in sterile phosphate buffered saline (PBS; pH 7.4) for 30 min at 37°C. The cultures were washed, then fixed and processed for immunofluorescence against TH or OX42 (see above).

CMTMR, MTGFM, and DHE-derived fluorescence was visualized with a laser scanning confocal microscope (TCS-SP2; Leica, Heidelberg, Germany), equipped with a 63 $\times$ oil immersion 1.4 numerical aperture (NA) objective. All cells were visualized at the same level of laser intensity, detector sensitivity, and pinhole size in order to ensure that fluorescence intensity could be compared among different coverslips and treatments. The images were saved as 8-bit TIFF files and the fluorescence intensity was evaluated by digital image processing with ImageJ software (NIH, Bethesda). The cytoplasm of dopaminergic or microglial cells was identified by TH or OX42 staining. A minimum of 50 TH-ir or OX42-ir fluorescent cells were counted in random visual fields to assess the intensity of DHE-derived or CMTMR or MTGFM fluorescence in cultures labeled for these markers. All measurements were corrected for background fluorescence.

Cell counting

TH-ir cells were counted in five randomly chosen longitudinal and transverse microscopic fields along

the diameter of the culture dish, away from the curved edge. The operator was blind to the treatment condition. The microscopic field was defined by a 0.5 $\times$ 0.5 cm reticule (i.e., 1.25 cm²). The average number of TH-positive cells in a control culture dish was 1,628 $\pm$ 76. The results from at least three separate experiments were recorded, with a minimum sample size of four dishes per group and per run. The results for each batch were normalized to the control group counts (i.e., expressed as a percentage of the control group counts) to counteract any variability among batches. Statistical differences between groups were tested as described below.

[³H] Dopamine uptake

The culture medium was removed completely and cultures were rinsed twice with 1 ml of uptake buffer (Krebs buffer+1.8 mM Cl₂Ca+25 mM D-glucose). Cells were then incubated for 30 min at 37°C with 1 ml of uptake buffer (containing 1 mM ascorbic acid and 100 μ M pargyline; pH 7.4) in the absence (untreated cultures) or presence of 10 μ M 5-HD. Uptake was initiated by addition of 20 nM [³H] dopamine ([2,5,6-³H] dopamine; 1 μ Ci, 12 Ci/mmol; Amersham Biosciences, GE Healthcare, Buckinghamshire, UK) in 20 μ l of Krebs buffer. Non-specific uptake values were defined in the presence of 10 μ M GBR 12935, a specific inhibitor of the dopamine transporter. Uptake was stopped after 30 min incubation at 37°C by removal of the incubation mixture and the cells were washed twice with cold Krebs buffer. Cells were lysed with 1 ml of 2 N NaOH for 30 min at room temperature, and the radioactivity incorporated into cells was measured by liquid scintillation spectrometry. Results are expressed as percentages of untreated control culture responses.

Statistical analysis

All data were obtained from at least three independent experiments and were expressed as means $\pm$ SEM. Two-group comparisons were analyzed by the Student's *t* test and multiple comparisons were analyzed by one-way ANOVA followed by Bonferroni's post hoc test. The normality of populations and homogeneity of variances were tested before each ANOVA. Differences were considered statistically significant at *p* <0.05. Statistical analyses were carried out with

SigmaStat 3.0 from Jandel Scientific (San Rafael, CA, USA).

Results

MitoK(ATP) channel inhibition blocks the enhancing effect of angiotensin II on dopaminergic neuron degeneration

Cultures treated with very low doses of 6-OHDA (10 μ M) showed a low non-significant decrease in the number of TH-ir neurons. However, the loss of dopaminergic neurons was significantly enhanced (loss of around 50% of dopaminergic neurons) by treatment with angiotensin II (100 nM). Treatment with the angiotensin II type 1 receptor antagonist ZD 7155 (6-OHDA+angiotensin+ZD) or the mitoK(ATP) channel inhibitor 5-HD (6-OHDA+angiotensin+5-HD) blocked the loss of dopaminergic neurons (Fig. 1a).

Cultures treated with low doses of MPP⁺ (0.25 μ M) showed a low although significant decrease (20–25% decrease) in the number of TH-ir neurons. Again, the loss of dopaminergic neurons was significantly enhanced (around 50–55% decrease) by treatment with angiotensin II (100 nM). Treatment with the angiotensin II type 1 receptor antagonist ZD 7155 or the mitoK(ATP) channel inhibitor 5-HD (MPP⁺+angiotensin+5-HD) blocked the enhancing effect of angiotensin II on the loss of dopaminergic neurons (Fig. 1b). No significant loss of dopaminergic neurons was observed after administration of angiotensin II alone or 5-HD alone or ZD 7155 alone (Fig. 1c). These data confirm the results of our previous studies showing that

Fig. 1 Effects of different treatments on the number of TH-ir cells. Treatment with very low doses of 6-OHDA (10 μ M; **a**) or MPP⁺ (0.25 μ M; **b**) induced a slight decrease (non-significant and significant, respectively) in the number of TH-ir neurons, which was significantly enhanced by angiotensin II (100 nM). The amplifying effect of angiotensin II was blocked by simultaneous treatment with the angiotensin II type 1 receptor antagonist ZD 7155 or with the mitoK(ATP) channel inhibitor 5-HD (10 μ M and 100 μ M). No significant effect was observed after treatment with angiotensin II or ZD 7155 or 5-HD alone (**c**). The data are expressed as percentages of the number of TH-ir cells obtained in the respective control cultures (100%). Data represent means $\pm$ SEM. * p <0.05 compared with control group (untreated cells), # p <0.05 compared with the group treated with 6-OHDA or MPP⁺ and angiotensin (one-way ANOVA and Bonferroni post hoc test). AII angiotensin II, TH tyrosine hydroxylase, ZD ZD 7155, 5-HD 5-hydroxydecanoic acid

angiotensin II increases the effect of low doses of dopaminergic neurotoxins via type 1 receptors, and also demonstrate that mitoK(ATP) receptor activation is involved in this effect (Fig. 2).

The results observed with 5-HD were confirmed with control cultures treated with the K(ATP) antagonist glibenclamide. Cultures treated with 6-OHDA + angiotensin II or MPP⁺ + angiotensin II and glibenclamide (10 μ M) contained significantly more TH-ir neurons than cultures treated with the neurotoxins and angiotensin alone (Fig. 3).

[³H] Dopamine uptake assay

The neurotoxins 6-OHDA and MPP⁺ are accumulated by the dopamine uptake system. It is therefore possible that changes in 6-OHDA- or MPP⁺-induced neurotoxicity were caused by increased or decreased uptake of the neurotoxin due to 5-HD-induced changes in the dopamine transport activity. In the present study, [³H] dopamine uptake was measured in the absence or presence of 5-HD (10 μ M). No significant changes ($p > 0.05$) in the uptake of [³H] dopamine were observed after treatment with 5-HD ($92.9 \pm 14.9\%$) in comparison with non-treated controls (100%).

MitoK(ATP) channel inhibition blocks the enhancing effect of angiotensin II on generation of intracellular superoxide/superoxide-derived ROS in dopaminergic neurons

Detection of superoxide anion and superoxide-derived ROS in cultured cells was achieved by treatment with dihydroethidium (DHE), which is oxidized to 2-hydroxyethidium and fluoresces red. Double labeling for TH and DHE-derived fluorescence revealed that treatment with very low doses of 6-OHDA (10 μ M) induced a slight significant increase in the intensity of DHE-derived fluorescence 3 h after treatment. The 6-OHDA-induced increase in DHE-derived fluorescence in dopaminergic neurons was significantly increased by administration of angiotensin II, and decreased to control levels after treatment with the mitoK(ATP) blocker 5-HD (6-OHDA + angiotensin + 5-HD). Administration of angiotensin II alone induced a slight but significant increase in the intensity of DHE-derived fluorescence. Treatment with 5-HD alone induced a small non-significant decrease in the

intensity of DHE-derived fluorescence with respect to controls (Figs. 4a and 6a–c).

Cultures treated with low doses of MPP⁺ showed similar results. Administration of angiotensin II significantly increased the intensity of DHE-derived fluorescence observed in dopaminergic neurons of control cultures or cultures treated with MPP⁺ alone, and the increase was blocked by treatment with 5-HD (MPP⁺ + angiotensin + 5-HD). Administration of angiotensin II alone induced a slight but significant increase in the intensity of DHE-derived fluorescence. Treatment with 5-HD alone induced a small non-significant decrease in the intensity of DHE-derived fluorescence with respect to controls (Fig. 4b).

MitoK(ATP) channel inhibition blocks the angiotensin II-induced decrease in mitochondrial inner membrane potential ($\Delta\psi_M$) in dopaminergic neurons

CMTMR (MitoTracker Orange) was used to estimate $\Delta\psi_M$. CMTMR enters mitochondria in proportion to the negative charge difference between the cytoplasm and the mitochondrial matrix and binds irreversibly to mitochondrial matrix thiols. Double labeling for TH and CMTMR revealed that treatment of cultures with angiotensin II induced a significant reduction in CMTMR fluorescence in dopaminergic neurons, which revealed a decrease in $\Delta\psi_M$ in these neurons. Treatment with the mitoK(ATP) blocker 5-HD inhibited the angiotensin II-induced decrease in CMTMR fluorescence (i.e., angiotensin-induced decrease in $\Delta\psi_M$) in dopaminergic neurons (Figs. 5a and 6d–l). Interestingly TH-ir neurons treated with angiotensin + 5-HD or with 5-HD alone showed higher levels of CMTMR fluorescence than TH-ir neurons from untreated cultures. This indicates that 5-HD treatment also blocked the effect of other factors (i.e., in addition to angiotensin II) that act on cultured dopaminergic neurons and decrease their $\Delta\psi_M$ (see “Discussion”).

Increases in CMTMR fluorescence may indicate an increase in $\Delta\psi_M$ or an increase in mitochondrial mass. In an attempt to differentiate between these possibilities, we used the probe MTGFM, an indicator of mitochondrial mass, and we observed that MTGFM fluorescence in the dopaminergic neurons remained unchanged 3 h after the corresponding treatment exposure (Figs. 5b and 6m–u). Furthermore,

Fig. 2 Photomicrographs of representative TH-ir cells from different experimental groups: a control culture (**a**), and cultures treated with a low dose of 6-OHDA (10 μ M; **b**), or 6-OHDA (10 μ M)+angiotensin (AII, 100 nM; **c**), or 6-OHDA+AII+5-HD (10 μ M; **d**), or AII alone (**e**), or 5-HD alone (**f**). AII

induced a significant increase in 6-OHDA neurotoxicity. This increase was blocked by treatment with the mitoK(ATP) channel inhibitor 5-HD. AII angiotensin II, 5-HD 5-hydroxydecanoic acid. Scale bar: 100 μ m

the hyperpolarizing effect of 5-HD was also confirmed in living cells in mesencephalic cell cultures using the JC-1 assay (Fig. 5c). Finally, the negatively

charged molecule 5-HD was unable to increase CMTMR or J-aggregate fluorescence in cells treated with protonophore CCCP. This revealed that the

Fig. 3 Effects of different treatments on the number of TH-ir cells. Treatment with very low doses of 6-OHDA (10 μ M; **a**) or MPP⁺ (0.25 μ M; **b**) induced a slight decrease (non-significant and significant, respectively) in the number of TH-ir neurons, which was significantly enhanced by angiotensin II (100 nM). The amplifying effect of angiotensin II was blocked by simultaneous treatment with the K(ATP) channel inhibitor glibenclamide (10 μ M), which confirmed the protective effect on dopaminergic degeneration observed with 5-HD. No significant effect was observed after treatment with glibenclamide alone. The data are expressed as percentages of the number of TH-ir cells obtained in the respective control cultures (100%). Data represent means $\pm$ SEM. * p < 0.05 compared with control group (untreated cells), # p < 0.05 compared with the group treated with 6-OHDA or MPP⁺ and angiotensin (one-way ANOVA and Bonferroni post hoc test). AII angiotensin II, TH tyrosine hydroxylase, Glib glibenclamide

increase in CMTMR or JC-1 retention is not due to non-specific effects and is dependent of $\Delta\psi_M$ (Fig. 5a, c).

Fig. 4 Intracellular levels of superoxide/superoxide-derived ROS estimated by treatment with dihydroethidium (DHE) in dopaminergic neurons (i.e., TH-ir cells). Administration of very low doses of 6-OHDA (10 μ M; **a**) or MPP⁺ (0.25 μ M; **b**) induced a significant (6-OHDA) or non-significant (MPP⁺) increase in the intensity of DHE-derived fluorescence. Treatment with 6-OHDA or MPP⁺+angiotensin II (AII; 100 nM) induced a significant increase in the intensity of DHE-derived fluorescence, and the increase was blocked by simultaneous administration of the mitoK(ATP) channel inhibitor 5-HD. Treatment with angiotensin II alone induced a significant increase in DHE-derived labeling. No significant effect was observed after treatment with 5-HD alone. Data are means $\pm$ SEM. * p < 0.05 compared with control group (untreated cells); # p < 0.05 compared with the group treated with 6-OHDA+AII or MPP⁺+AII (one-way ANOVA and Bonferroni's post hoc test). AII angiotensin II, DA dopaminergic, 5-HD 5-hydroxydecanoic acid

Fig. 5 **a** Treatment of cultures with AII induced a significant reduction in CMTMR fluorescence (i.e., $\Delta\psi_M$) in dopaminergic neurons. Treatment with the angiotensin type 1 receptor antagonist ZD 7155 or the mitoK(ATP) channel blocker 5-HD inhibited the angiotensin II-induced decrease in CMTMR fluorescence. TH-ir neurons treated with AII+5-HD or 5-HD alone (i.e., without AII) showed higher levels of CMTMR fluorescence than TH-ir neurons in non-treated cultures. This suggests that 5-HD treatment may also block the effect of additional factors that decrease the $\Delta\psi_M$ in cultures. Furthermore, 5-HD was unable to increase CMTMR fluorescence in cells treated with protonophore CCCP, which revealed that the increase in CMTR retention is not due to non-specific effects and is dependent on $\Delta\psi_M$. **b** MitoTracker Green FM (MTGFM) labeling analysis showed that changes in CMTMR fluorescence were not caused by changes in total mitochondrial mass. **c** The effects of 5-HD were also confirmed in living cells in mesencephalic cell cultures using the JC-1 assay. 5-HD was also unable to increase J-aggregate fluorescence in cells treated with CCCP. ^{*} $p < 0.05$ compared with control group (untreated cells), [#] $p < 0.05$ in comparison with the groups treated with 5-HD+AII or 5-HD alone, ^s $p < 0.05$ (one-way ANOVA and Bonferroni's post hoc test). AII angiotensin II, CCCP carbonyl cyanide *m*-chlorophenylhydrazide, DA dopaminergic, ZD ZD 7155, 5-HD 5-hydroxydecanoic acid

Role of microglial cells: mitoK(ATP) channel inhibition in neuron-enriched cultures

Involvement of microglial cells in the above mentioned effects of angiotensin II and 5-HD was studied in primary mesencephalic cultures in which microglial cells were identified with OX-42, and levels of superoxide/superoxide-derived ROS were estimated with DHE (i.e., double OX42-DHE labeling). Treatment of cultures with 10 μ M 6-OHDA alone or angiotensin II alone did not induce a significant increase in the intensity of DHE-derived fluorescence in microglial cells. However, simultaneous treatment with 10 μ M 6-OHDA+angiotensin II significantly increased the intensity of DHE-derived fluorescence induced by 6-OHDA alone or angiotensin II alone, which was reduced to control levels by treatment with 5-HD (i.e., 6-OHDA+angiotensin+5-HD; Fig. 7a).

Neuron-enriched cultures in which glia was eliminated with Ara C were used to confirm the potential role of microglial cells in the observed enhancing effect of angiotensin II and the protective effect of 5-HD on dopaminergic neuron degeneration. In the absence of glia, very low doses of 6-OHDA (10 μ M) or 6-OHDA+angiotensin II did not induce a significant increase in levels of superoxide/superoxide-derived ROS (i.e., intensity of DHE-derived fluores-

Fig. 6 Double labeling for TH (green in **a**, **d**, **g**, **j**; red in **m**, **p**, **s**) and DHE-derived fluorescence (red; **a–c**), or CMTMR (MitoTracker Orange, red; **d–f**), or MTG (MitoTracker Green, green; **m–u**). Cultures treated with 6-OHDA (10 μ M)+angiotensin II (AII; 100 nM) displayed intense DHE-derived fluorescence (red) in dopaminergic neurons (**b**), which was higher than in control cultures (**a**) or in those cultures treated with 6-OHDA+AII and 5-HD (**c**). Treatment of cultures with AII (100 nM) alone induced a significant reduction in CMTMR fluorescence (i.e., $\Delta\Psi_M$; red) in dopaminergic neurons (**g–i**; green) with respect to controls (**d–f**). The decrease was inhibited by treatment with 5-HD (**j–l**). However, treatment of cultures with AII (100 nM) alone or AII+5-HD did not induce significant changes in MTG fluorescence (i.e., mitochondrial mass; green) in dopaminergic neurons (**p–u**; red) with respect to controls (**m–o**). Cell nuclei were labeled with Hoechst 33342 (blue) in (**o**), (**r**), and (**u**). AII angiotensin II, MTO MitoTracker Orange, MTG MitoTracker Green, DHE dihydroethidium, TH tyrosine hydroxylase. Scale bar: 75 μ m (**a–c**) and 50 μ m (**d–u**)

cence; Fig. 7b). Moreover, treatment with very low doses of 6-OHDA (10 μ M) induced a non-significant

decrease in the number of TH-ir neurons in the absence of glia. Interestingly, angiotensin II adminis-

Fig. 7 Involvement of microglial cells in the effects of angiotensin II (AII) and 5-HD was studied in primary mesencephalic cultures in which microglial cells were identified with OX-42 (**a**) and in neuron-enriched cultures (i.e., treated with Ara C; **b**, **c**). **a** Simultaneous treatment with 10 μ M 6-OHDA+AII significantly increased the intensity of DHE-derived fluorescence induced by 6-OHDA alone or AII alone in microglial cells, which was reduced to control levels by treatment with 5-HD (i.e., 6-OHDA+AII+5-HD). In the absence of glia, treatment with very low doses of 6-OHDA (10 μ M) or 6-OHDA+AII did not induce significant increase in intracellular levels of superoxide/superoxide-derived ROS in dopaminergic neurons (**b**), or a significant decrease in the number of TH-ir neurons (**c**), and 5-HD administration did not induce any significant change. However, higher doses of 6-OHDA (30 μ M) induced a significant loss of dopaminergic neurons, which was blocked by 5-HD (**c**). The data are expressed as percentages of the respective control cultures (100%). Data represent means $\pm$ SEM. ^{*} p <0.05 compared with control group, [#] p <0.05 compared with group treated with 6-OHDA (30 μ M) in (**c**) (one-way ANOVA and Bonferroni post hoc test). AII angiotensin II, DA dopaminergic, TH tyrosine hydroxylase, 5-HD 5-hydroxydecanoic acid

observed after treatment with 5-HD (Fig. 7c). This indicates that glial cell factors (e.g., ROS from microglia) play a major role in the effect of angiotensin II in increasing dopaminergic neuron death. In order to determine if mitoK(ATP) channels in dopaminergic neurons are involved in dopaminergic degeneration induced by higher levels of ROS than those induced by 10 μ M 6-OHDA in the absence of glial cells, neuron-enriched cultures were treated with higher doses of 6-OHDA. Doses of 30 μ M 6-OHDA induced a significant loss of dopaminergic neurons (decrease of approximately 40%), which was blocked by inhibition of neuronal mitoK(ATP) channels with 5-HD (Fig. 7c).

Discussion

Several studies in different tissues have shown that normal aging is associated with a pro-inflammatory, pro-oxidant state that may favor an exaggerated response to injury and degenerative diseases (Choi et al. 2010; Csiszar et al. 2003; Ungvari et al. 2004) and that increased activity of local angiotensin via angiotensin II type 1 receptors is involved in age-related degenerative diseases (Basso et al. 2005; Mukai et al. 2002). In agreement, we have recently observed an age-related increased angiotensin activity in the nigra that may increase the vulnerability of

tration did not induce any additional loss of dopaminergic neurons, and no significant changes were

dopaminergic neurons to additional damage, and the risk of Parkinson's disease with aging (Villar-Cheda et al. 2010b). Furthermore, the interaction between renin–angiotensin system and dopaminergic system is of particular interest. Several studies have shown an important interaction between dopamine and angiotensin II receptors in peripheral tissues, particularly as regards regulating renal sodium excretion and cardiovascular function (Gildea 2009; Zeng et al. 2006; Khan et al. 2008). Recent evidence suggests that dopaminergic and renin–angiotensin systems directly counterregulate each other in renal cells (Gildea 2009) and also in the nigrostriatal system (Villar-Cheda et al. 2010a), and that abnormal counterregulation between dopamine and angiotensin II plays an important role in degenerative changes (Joglar et al. 2009; Li et al. 2008; Rodriguez-Pallares et al. 2008). Interestingly, several studies have shown that there is an aging-related decrease in dopamine release, which results in a progressive decrease in motor activity (Collier et al. 2007; Gerhardt et al. 2002), and may be responsible for the above mentioned aging-related increase in angiotensin activity (Villar-Cheda et al. 2010a,b).

In cardiovascular and renal tissues, angiotensin II induces oxidative stress and inflammation via activation of the NADPH oxidase (Qin et al. 2004; Seshiah et al. 2002; Touyz et al. 2002). Similarly, we have recently shown that the effect of low/sublethal doses of neurotoxins on dopaminergic cell loss is amplified by angiotensin II via type 1 receptors and protein kinase C, leading to activation of the microglial NADPH oxidase and exacerbation of the glial inflammatory response, which increases ROS released by in microglia and dopaminergic neurons (Joglar et al. 2009; Rey et al. 2007; Rodriguez-Pallares et al. 2008). However, recent studies suggest that angiotensin II may stimulate not only cytosolic (i.e., via NADPH oxidase activation) but also mitochondrial-ROS generation in several tissues (de Cavanagh et al. 2007; Zhang et al. 2007), and numerous studies have shown that mitochondria play a major role in generation of oxidative stress, dopaminergic neuron degeneration, and aging (de Cavanagh et al. 2007; Schapira 2008). This is consistent with recent studies that suggest cross-talk signaling between both the cytosolic NADPH oxidase and mitochondria in several types of cells, and that mitoK(ATP) play a major role in this interaction (Brandes 2005; Daiber 2010; Dikalova et al. 2010; Doughan et al. 2008).

Therefore, NADPH oxidase may lead to mitoK(ATP) channel opening, which play a major role in modulating intracellular ROS (Andrakhiv et al. 2006; Costa and Garlid 2008; Mattson and Liu 2003) via regulation of the formation/release of ROS from the mitochondria, and integration of signals from diverse sources (Facundo et al. 2007; Fornazari et al. 2008; Oldenburg et al. 2002). ROS open up mitoK(ATP), and mitochondrial ROS production can be stimulated by the opening of mitochondrial mitoK(ATP) channels (Costa and Garlid 2008; Kimura et al. 2005b; Zhang et al. 2002).

In accordance with the above mentioned studies, the present results reveal that activation of mitoK(ATP) channels may play important role in the effects of angiotensin II on the dopaminergic system via NADPH oxidase activation. Inhibition of mitoK(ATP) channels with 5-HD blocked the increase in dopaminergic cell death induced by angiotensin II, as well as the angiotensin II-induced increase in superoxide/superoxide-derived ROS levels in dopaminergic neurons, and the angiotensin II-induced decrease in mitochondrial inner membrane potential ($\Delta\psi_M$) in dopaminergic neurons. 5-HD is the most widely used specific inhibitor of mitoK(ATP) channels, and several studies have shown that 5-HD (10 μ M) blocks the mitoK(ATP) channels without any effect on cell membrane K(ATP) channels (Costa and Garlid 2008; Garlid et al. 1997; McCullough et al. 1991; Zhang et al. 2007). Doses up to 500 mmol/l have been reported to inhibit selectively mitoK(ATP) channels without affecting surface K(ATP) currents (Sato et al. 2000; Hu et al. 1999). However, we did not observe significant differences between the effect of 10 μ M 5-HD and 100 μ M 5-HD, and high doses of 5-HD may have non-specific side effects independent of any action on mitoK(ATP), as reported for mitoK(ATP) openers, which have been observed to act as channel closers at high concentrations (Garlid 2000; Wu et al. 2006). In the present study, we therefore used the lowest dose of 5-HD (10 μ M), which proved effective at inhibiting the effects of angiotensin II. Furthermore, it is known that 5-HD does not exert any radical scavenging activity (Kimura et al. 2005a, b), and the results cannot be attributed to a 5-HD-induced decrease in 6-OHDA uptake, as revealed in the present study by the [^{3} H] dopamine uptake assay. In addition, treatment with a second K(ATP) inhibitor (i.e., glibenclamide) confirmed the protective effect observed with 5-HD.

The cross-talk signaling between NADPH oxidase and mitochondria means that angiotensin II stimulation induces NADPH activation, which leads to opening of mitoK(ATP), depolarizes mitochondrial potential, and amplifies ROS generation from mitochondria (Brandes 2005; Kimura et al. 2005a, b; Zhang et al. 2007), but also that mitochondrial ROS have a significant upstream impact on increasing NADPH oxidase activity (Li et al. 2001; Wosniak et al. 2009). Thus, a vicious cycle consisting of mitochondrial and NADPH oxidase-derived ROS formation with each stimulating the other in a positive feedback fashion leads to increased cytoplasmic ROS levels (Daiber 2010; Dikalova et al. 2010). This has clearly been observed in recent studies in endothelial cells, which showed that inhibition of NADPH activity by apocynin or deletion of NADPH oxidase subunits prevented mitochondrial impairment and attenuated mitochondrial superoxide production via mitoK(ATP) channels (Doughan et al. 2008); in addition, mitochondrial superoxide stimulated extra-mitochondrial NADPH oxidase activity, which was blocked by SOD2 (manganese-containing mitochondrial superoxide dismutase), the mitochondria-targeted superoxide dismutase mimetic mitoTEMPO or the inhibitor of mitoK(ATP) channels 5-HD (Dikalova et al. 2010).

K(ATP) channels were originally discovered in the heart, although are particularly abundant in the CNS, and occur at highest levels in the substantia nigra and striatum (Busija et al. 2004; Zini et al. 1993). Brain mitochondria contain seven times more mitoK(ATP) channels per milligram of mitochondrial protein than liver or heart (Bajgar et al. 2001; Bednarczyk 2009). It is therefore possible that, as suggested for peripheral tissues (Facundo et al. 2007; Fornazari et al. 2008; Oldenburg et al. 2002), these channels provide in dopaminergic neurons a convergent target that could integrate ROS induced by low levels of neurotoxins (6-OHDA or MPP⁺) with angiotensin/NADPH oxidase-induced ROS to increase dopaminergic neuron oxidative stress and cell death. It may be speculated that low doses of neurotoxins, or other factors in PD, may generate low levels of ROS by several mechanisms that are insufficient to induce dopaminergic cell death. However, the low levels of ROS, together with those derived from angiotensin-induced NADPH oxidase activation, may act as a trigger to activate mitoK(ATP) channels in the

mitochondrial inner membrane, thereby enhancing ROS production and subsequent progression of dopaminergic cell degeneration. This is also supported by our previous studies that observed that the effects of exogenous ROS generated by autooxidation of 6-OHDA are decreased by inhibition of mitoK(ATP) (Rodriguez-Pallares et al. 2009). However, it has also been reported that mitoK(ATP) opens inhibit rotenone-induced microglial activation and neuroinflammation (Zhou et al. 2008). The discrepancies may be related to different experimental conditions. MitoK(ATP) openers have been reported to act as channel closers at high concentrations (Wu et al. 2006). It has also been suggested that opening of mitoK(ATP) may have two distinct consequences, depending on the underlying bioenergetic state (Garlid et al. 2003). When the $\Delta\psi_M$ is high, as in normoxic and resting cells, opening of channels causes net K⁺ influx and matrix alkalinization with a consequent rise in mitochondrial ROS production. When $\Delta\psi_M$ is depressed, as occurs during ischemia or treatment with the complex I blocker rotenone, mitoK(ATP) opening may add a parallel K⁺ conductance that counteracts the decrease in K⁺ influx and matrix contraction that otherwise occur, and therefore mitoK(ATP) opening may maintain constant volume of the mitochondrial matrix and the intermembrane space (Bajgar et al. 2001; Garlid et al. 2003; Kowaltowski et al. 2001). It is interesting to note that activation of mitoK(ATP) channels and ROS production lead to delayed protection against subsequent intense and potentially lethal insults such as ischemia by activation of adaptive cell responses in several types of cells (i.e., preconditioning; Kis et al. 2003; Oldenburg et al. 2002). Conversely, the present study shows that ROS induced by activation of mitoK(ATP) channels contribute to dopaminergic cell death. This is consistent with the results of previous studies that have shown that dopaminergic neurons function in a “near-compromised” state and are more vulnerable to oxidative stress and to mitochondrial complex I inhibition than other neurons and cell types (Bertarbet et al. 2000; Hirsch et al. 1997; Obeso et al. 2010). Therefore, activation of mitoK(ATP) channels and increased ROS levels, which may be useful for preconditioning and delayed protection in other cell types, may overwhelm the defense mechanisms of dopaminergic neurons. This may also be supported by the hyperpolarizing effect of 5-HD observed in

TH-ir neurons treated with angiotensin+5-HD or with 5-HD alone, which suggests that 5-HD treatment may also block the effect of other factors (i.e., in addition to AII) that act on cultured dopaminergic neurons and decrease their $\Delta\psi_M$. The hyperpolarizing effect of 5-HD may be related to experimental conditions and the cell type, and has been also observed in other studies (Valero et al. 2008). However, this observation is particularly interesting in the case of dopaminergic neurons since a number of previous studies have shown that the dopaminergic neurons have high levels of ROS. A number of factors are thought to be involved, including increased iron content, reduced antioxidant capacity or factors associated with the dopamine synthesized, released, and metabolized in these neurons (Fahn and Cohen 1992; Olanow 1990). The protective defense mechanisms for dopaminergic neurons may be overwhelmed by additional deleterious factors in neurons already subjected to dopamine-derived toxicity thus leading to dopaminergic neuron death (i.e., a “synergistic effect hypothesis”). In the present and previous studies, we suggest that the brain renin–angiotensin system plays a major role in this process since major sources of ROS such as NADPH oxidase and mitochondria are enhanced by renin–angiotensin system activation.

As angiotensin II type 1 receptors and NADPH oxidase have both been observed in dopaminergic neurons and microglial cells (Joglar et al. 2009; Rodriguez-Pallares et al. 2008), it is possible that neurotoxins may interact synergistically with ROS derived from either neuronal or microglial NADPH oxidase activation to activate mitoK(ATP) channels and enhance ROS production. In microglial cells (i.e., OX-42 ir cells), we observed that angiotensin II significantly increased levels of ROS induced by very low doses of neurotoxins (10 μ M 6-OHDA), which is inhibited by 5-HD. This suggests a role for microglial mitoK(ATP) channels in angiotensin-induced amplification of ROS release and microglial activation. In inflammatory cells such as microglia, NADPH oxidase activation leads to ROS production that serves dual functions. Firstly, high concentrations of ROS are released extracellularly to kill invading microorganisms or cells (Babior 1999, 2004). Secondly, low levels of intracellular ROS act as a second messenger in a considerable number of signaling pathways involved in the inflammatory response (Mattson and Maudsley 2009; Qin et al. 2004; Touyz et al. 2002).

The present results suggest that mitoK(ATP) play a major role in these mechanisms.

The possible neuronal origin of angiotensin/NADPH oxidase-induced ROS was studied using neuron enriched cultures, in which very low doses of neurotoxin (10 μ M 6-OHDA) did not induce a significant loss of dopaminergic neurons, and this effect was not significantly modified by angiotensin II or angiotensin+5-HD. This suggests that a direct effect of angiotensin II on neuronal NADPH oxidase activation elicits low levels of ROS which, acting together with those derived from 10 μ M 6-OHDA, are insufficient to induce significant mitoK(ATP) channel activation and/or dopaminergic degeneration. This is consistent with data showing that in non-inflammatory cells, such as neurons, the NADPH oxidase produces only low rates of ROS for signaling function (Babior 1999, 2004). However, we also demonstrated that neuronal mitoK(ATP) channels are involved in dopaminergic cell death when channels are activated by higher levels of ROS such as those derived from 30 μ M 6-OHDA in the present experiments in neuron-enriched cultures (dopaminergic cell death was blocked in the presence of 5-HD), or those derived from activated microglia in primary (i.e., neuron/glia) cultures.

In summary, the data suggest that angiotensin II, via type I receptors, activates microglial NADPH oxidase (i.e., NOX2, phagocytic oxidase, PHOX) that induces mitoK(ATP) opening and increases mitochondrial-derived superoxide, which stimulates extramitochondrial NADPH oxidase activity in a feed-forward fashion and constitutes a vicious cycle leading to enhanced microglial activation and extracellular release of ROS. MitoK(ATP) channels of dopaminergic neurons may respond to high levels of ROS derived from the angiotensin-related microglial activation and increase neuronal NADPH oxidase activity. Furthermore, neuronal mitoK(ATP) channels may provide a convergent target to integrate different sources of neuronal ROS (ROS derived from 6-OHDA or MPP⁺, or from neuronal NADPH oxidase activated by angiotensin via neuronal type 1 receptors in the present experiments, or from other factors in Parkinson’s disease) and ROS from activated microglia to induce dopaminergic cell death. In conclusion, the present results reveal that mitoK(ATP) channels may play a major role in the enhancing effect of angiotensin II on dopaminergic degeneration triggered

by low/sublethal doses of dopaminergic neurotoxins, and possibly in the synergistic interaction of several intra- and/or extraneuronal factors to increase levels of oxidative stress and dopaminergic cell death in PD. This is particularly interesting since aging-related increase in nigral renin–angiotensin system activity may constitute a major factor in the increased risk of PD with aging. Furthermore, the present study provides additional data for considering brain renin–angiotensin system and mitoK(ATP) channels as potential targets for neuroprotection in PD and aging.

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