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Angiotensin II protects against α -synuclein toxicity and reduces protein aggregation in vitro

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Abstract

In this study, we examined the effects of angiotensin II (AngII) in a genetic *in-vitro* PD model produced by α -synuclein (α -syn) overexpression in the human neuroglioma H4 cell line. We observed a maximal decrease in α -syn-induced toxicity of 85% and reduction in inclusion formation by 19% when cultures were treated with AngII in the presence of the angiotensin type 1 (AT1) receptor antagonist losartan and AT2 receptor antagonist PD123319. When compared to AngII, the AT4 receptor agonist AngIV was moderately effective in protecting H4 cells against α -syn toxicity and did not significantly reduce inclusion formation. Here we show that AngII is protective against genetic, as well as neurotoxic models of PD. These data support the view that agents acting on the renin-angiotensin-system (RAS) may be useful in the prevention and/or treatment of Parkinson disease.

Keywords

Parkinson's disease; inclusion; Renin-Angiotensin-System; synuclein; Synphilin-1; angiotensin; AT receptor; losartan; PD123319

Introduction

Alpha-synuclein (α -syn) plays a central role in the pathogenesis of Parkinson's disease (PD), a common and debilitating neurodegenerative disorder [1,2]. A mutation in α -syn was the first genetic defect identified in PD [3]. Subsequent studies have shown that α -syn gene duplications also cause PD and that aggregates of α -syn are present in nearly all cases of sporadic PD [4, 5,6,7]. The mechanisms by which mutations in α -syn lead to PD, and the significance of changes in α -syn levels is unclear. The discovery of the central role of this protein has led to the development of a number of novel models of PD, including cellular systems, drosophila, and mouse and non-human primate models.

The renin-angiotensin-system (RAS) has long been known for its role in regulating blood pressure, salt and water homeostasis and vascular tone [8,9]. The primary agonist of the RAS

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is the octapeptide angiotensin II (AngII). AngII mediates its cardiovascular effects primarily through two 7-transmembrane G protein coupled receptors, angiotensin type 1 (AT1) and angiotensin type 2 (AT2) [10]. Recent studies have revealed that the RAS is active in the brain as well as in the circulatory system, and may have an important role in neurodegeneration [11,12]. Other studies have shown that agents acting on the RAS are effective in stroke as well as models of Alzheimer disease. [13,14,15]. In PD models, RAS antagonists of the AT1 receptor and inhibitors of ACE can significantly reduce the loss of dopamine neurons in both *in vitro* and *in vivo* neurotoxin based models including MPTP, 6-hydroxydopamine and rotenone [16,17,18,19,20,21,22,23,24,25,26,27,28]. While neurotoxin-based models of PD have been crucial in our current understanding of how dopamine neurons are damaged, they do not recapitulate all the hallmarks of the human disease condition [29,30]. Genetic models of PD have provided a different perspective in understanding the etiology of PD and more specifically, on how α -syn and protein aggregation can be devastating to dopamine neurons [31,32,33]. In this study, we determined whether manipulation of different components of the RAS could mediate protection against a genetically based *in-vitro* PD model produced by α -syn overexpression. Our data show that activation of the RAS can reduce both the formation of α -syn aggregates and the toxicity of α -syn, further validating the potential of drugs acting on the RAS and angiotensin as neuroprotective therapy for PD.

Materials and Methods

Cell culture and transfections

Human H4 neuroglioma cells (HTB-148 - ATCC, Manassas, VA, USA) were maintained in OPTI-MEM (Life Technologies, Grand Island, NY, USA) supplemented with 10% fetal bovine serum and maintained at 5% CO₂/37° C. Cells were passaged 24 h prior to transfection and plated in 24-well plates, 60-mm dishes or 4-chamber slides at a density of 50% confluency. Cells were transfected with equimolar ratios of plasmids using Superfect (Qiagen, Chatsworth, CA, USA) according to the manufacturer's instructions.

Plasmid construction

The constructs for human wild type (WT) untagged α -syn and its C-terminal tagged version (referred to as Syn-T) and synphilin-1 have been described previously [34,35].

α -Synuclein toxicity assay and cell treatment

Toxicity was analyzed 24 h after transfection (WT α -syn) by measuring the release of adenylate kinase from damaged cells using the ToxiLight™ kit (Cambrex, Walkersville, MD) according to the manufacturer's protocol. In short, cells were transfected as described above, grown for 2 hours in a 24-well plate, and treated with AngII (100 nM) or Ang IV (100 nM-1 μ M) for the duration of the experiment [23,24]. In an effort to determine AT receptor subtype specific activity, 15 min prior to AngII or AngIV treatments the AT1 receptor specific antagonist losartan (1 μ M) and/or the AT2 receptor specific antagonist PD123319 (1 μ M) were added to the cultures [23,24]. 24 hours post-treatment, 50 μ l of medium were taken from each well and transferred into a 96-well white plate. 100 μ l of ToxiLight™ reagent were added at one second interval, into each well, and the plate was incubated in the dark for 5 min. Luminescence was read with a Wallac Victor2 plate reader.

Immunohistochemical analysis

To determine AT receptor subtypes, a polyclonal (rabbit) anti-AT1 (1:50), a polyclonal (rabbit) anti-AT2 (1:100) antibody (Santa Cruz Biotechnology, Inc, Santa Cruz, CA) and a polyclonal (rabbit) anti-AT4 (1:100) antibody (Chemicon International, Temecula, CA) were used. Secondary antibodies used were the Alexa 495-conjugated (goat) anti-rabbit IgG (1:1000),

(Molecular Probes, Eugene, OR). For studying α -syn aggregation, cells were stained with a mouse anti- α -synuclein (1:1000) antibody (BD Transduction Laboratories, USA). A secondary Alexa488-conjugated goat anti-mouse (1:300) antibody (Molecular Probes, Eugene, OR, USA) was used. Stained cultures were visualized by fluorescence microscopy using a Leica confocal microscope.

α -Synuclein inclusion analysis and quantification

The α -syn inclusion assay has been described previously [34,35,36]. Cells were transfected using Superfect (Qiagen, Chatsworth, CA, USA) using equimolar ratios of the Syn-T and Synphilin-1 plasmids for co-transfections. Cells containing α -syn positive inclusions were assessed using a fluorescent microscope. Slides were counted by a blinded observer. Any cell which showed any α -syn immunostaining was considered a transfected cell, while any cell which demonstrated any number or size of inclusions detectable at 20x was considered an inclusion positive cell. The number of transfected cells containing inclusions was expressed as a percentage of total transfected cells. An average transfection yielded approximately 1500 transfected cells/well.

SDS-PAGE and immunoblotting

Cells were plated in 60 mm dishes 24 h prior to transfection. Transfections and drug treatments were performed as described above. Protein concentration was estimated using the BCA method and the appropriate volume of each sample were subjected to SDS-PAGE. Protein was identified by the addition of the primary antibody anti- α -synuclein (1:500) (BD Transduction laboratories), anti-V5 (for synphilin-1)(1:1000) (Abcam), or anti-actin (1:1000) (Sigma) and their appropriate secondary antibodies IRDye 800 anti-rabbit or anti-mouse (Rockland Immunochemicals, Gilbertsville, PA, USA) (1:3000) or Alexa-680 anti-rabbit or anti-mouse (Molecular Probes, Eugene, OR, USA) (1:3000). Immunoblots were processed and quantified using the Odyssey infrared imaging system (Lycor, Lincoln, NE, USA).

Statistical analysis

Data were analyzed using Prism software. Statistical significance was determined by one-way ANOVA, followed by post-hoc Newman-Keuls multiple comparison test. Differences in mean values were considered significant at $p < 0.05$. All data were obtained from at least three independent experiments and are represented as a mean + SEM.

Results

H4 cells express AT1, AT2 and AT4 receptor proteins

H4 cells were fixed in 4% paraformaldehyde, permeabilized and then immunostained with an anti-AT1, AT2 or AT4 receptor antibody and appropriate secondary antibodies. Immunostaining for each of the three proteins was detected in the H4 cells (Fig1). The proteins were localized to puncta within the cytoplasm of the cells as well as along the cell membranes. In addition, there was a variable degree of staining, most obvious with the AT1 antibody, faint with the AT4 antibody, and intermediate in intensity with the AT2 antibody.

AngII reduces the toxicity of α -synuclein, and the effect is enhanced by blockade of AT1 receptors

H4 cells were transfected with wild type α -syn or empty vector control and incubated with AngII (100 nM). After 24 hr of α -syn overexpression, cell culture media was harvested and assayed for adenylate kinase activity as a measure of cell death. Maximal toxicity was determined in H4 cells transfected with a plasmid containing wild type α -syn, after subtracting the background adenylate kinase activity observed in H4 cells that were transfected with a

control plasmid. Treatment with AngII (100 nM) reduced the toxicity of α -syn by $39.8 \pm 5\%$ (Fig2). Because the effect of AngII in neurotoxin models was enhanced by selective blockade of AT1 receptors in previous studies [24,25,26,27,28], we examined the effect of AngII with or without the AT1 receptor antagonist, losartan (10 nM-10 μ M). Losartan further enhanced the protective effect of AngII with maximal protection observed with 100 nM AngII in combination with 1 μ M losartan, producing a $66.8 \pm 11\%$ reduction in the release of adenylate kinase activity compared to α -syn alone (Fig2A).

Ang II protection is not mediated through the AT1 or AT2 receptor

To determine if AngII protection was AT receptor specific, H4 cells were exposed to combinations of the AT1 receptor antagonist losartan (1 μ M) and AT2 receptor antagonist PD123319 (1 μ M) in the presence or absence of exogenous AngII (100 nM). Maximal protection was observed when H4 cells were pretreated with both AT1 and AT2 receptor antagonists in the presence of exogenous AngII, showing a $85 \pm 12\%$ decrease in adenylate kinase release when compared α -syn alone transfected cells (Fig 2B).

AngIV moderately protects against α -synuclein toxicity

Because AngII was protective, but the effect did not appear to be mediated by either the AT1 or AT2 receptors, we tried to determine if this protection was attributable to actions of AngII at the AT4 receptor. We assessed whether the AT4 receptor agonist angiotensin IV (Ang IV) was more effective in protecting against α -syn toxicity. Early studies have identified the AT4 receptor as a high-affinity binding site for AngIV [28]. AngIV (100nM-1 μ M) moderately protected H4 cells against α -syn toxicity when compared to AngII (Fig2C). In the presence of the both AT receptor antagonists, AngIV showed a statistically significant but reduced protection against α -syn toxicity when compared to the AngII plus antagonists.

AngII but not AngIV prevents α -synuclein inclusions with antagonism of both the AT1 and AT2 receptors

In addition to altering cell viability, treatment with AngII also reduced the formation of visible aggregates of α -syn in transfected cells. The effect of AngII alone was modest and did not reach statistical significance (Fig3A), but aggregates were reduced by 17-19% ($p < 0.05$) in the presence of AngII and either the AT1 receptor antagonist losartan or the AT2 receptor antagonist PD123319 (Fig3B). AngIV, however, failed to significantly reduce the number of cells with α -syn inclusions, even in the presence of the AT receptor antagonists (Fig3B).

Angiotensin II and losartan treatments do not reduce expression levels of α -synuclein and synphilin-1

To rule out the possibility that the observed protection against α -syn toxicity and aggregation was a result of treatment-induced reduction in plasmid expression, we determined the expression levels of α -syn and synphilin-1 in transiently transfected H4 cells by through western blotting (Fig4). Densitometry measurements of protein bands corresponding to α -syn and synphilin-1 were normalized to actin expression to control for loading. We found no significant difference in expression levels following any of the treatments (data not shown). We conclude that the AngII and the angiotensin receptor antagonists do not interfere with the expression of the transfected plasmids.

Discussion

With the discovery of the local brain RAS, this system has emerged as an important target in neurodegenerative diseases. Recent studies in stroke models showed that ACE inhibitors, as well as AT1 receptor antagonists, can reduce ischemic injury and recovery time in vivo and in

vitro [38,39]. In addition, the brain RAS has been suggested to have a protective role against Alzheimer's disease because ACE can degrade beta amyloid protein [40,41].

While most of the actions of the RAS have been attributed to the activity of the AT1 and AT2 receptors, recent studies identified the novel AT4 receptor [42] which may be involved in memory formation, based on results showing improved learning in rats in water maze tests [43,44]. Because of the potential function of the AT4 receptor in memory it may be implicated in the brain RAS and, therefore, in Alzheimer's disease. The data described here further support that the brain RAS is an important target for treatment of PD. In neurotoxin-based PD models the AT1 receptor antagonist losartan prevents the loss of dopamine neurons in both primary ventral mesencephalic cultures as well as in the substantia nigra pars compacta (SNpc) of mice [45,46]. ACE inhibitors can also protect dopamine neuronal loss in vitro and in vivo [26,27, 28]. While neurotoxin models have proved useful, they do not recapitulate all the hallmarks of the human disease and their predictive value with respect to efficacy in clinical treatment is uncertain. It is important, therefore, to examine the potential of agents in both genetic-based and in neurotoxin models. Here we show that overexpressing wild type α -syn in H4 cells is toxic and that AngII in the presence of antagonists to the AT1 and AT2 receptors act synergistically to reduce the α -syn-induced toxicity. This finding differs from the result of earlier studies in neurotoxin models, where AngII mediated neuroprotection primarily occurs with inhibition of the AT1 receptor [45,46,47]. Because we observed a synergistic protective effect with losartan and PD123319 in the α -syn based model, these results suggest that AngII may be acting through a non-AT1 or AT2 receptor mechanism, and point to the potential involvement of the AT4 receptor. We used AngIV as an agonist for the AT4 receptor, and found that AngIV while moderately protected H4 cells from α -syn toxicity, it was significantly lower than that of AngII, even in the presence of inhibitors of the AT receptors. This data is not consistent with a protective effect of AT4 receptor but rather of a potential non-AT receptor mechanism. We also examined the effect of AngII and AngIV on another measure of α -syn toxicity, the formation of cellular aggregates. AngII, in the presence of AT antagonists, significantly reduced the formation of aggregates. However, AngIV failed to significantly reduce α -syn aggregation even in the presence of AT receptor antagonists. Thus, the RAS can modulate the biology of α -syn in the brain with respect to aggregation and cytotoxicity. Interestingly, previous studies have suggested that aggregation is not necessarily associated with cytotoxicity of α -syn, and may not be the best measure of the effectiveness of neuroprotective treatments [35]. Importantly, antagonists of the RAS, particularly inhibitors of AT1 receptors, are already widely used in humans for treatment of hypertension, which makes them strong candidates for further study. The data presented in this study provide a further basis for examining whether such modulators of the RAS are useful in the prevention or treatment of PD.

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Figure 1.
Immunohistochemical screening of AT receptors in H4 neuroglioma cells. Cultures were stained with antibodies specific for the AT1, AT2 and AT4 receptors and visualized by fluorescent secondary antibody Alexa 488 using confocal microscopy with 60x objective.

Figure 2.

Quantitative measurement of α -synuclein-induced adenylate kinase release (cell death). A) Dose response of the AT1 receptor antagonist losartan (10 nM-10 μ M) in α -syn transfected cells in the presence of AngII (100 nM). (*) Shows significance from α -syn alone control with $p < 0.05$ using One-way ANOVA with Newman Keuls post hoc analysis. Data is represented as $\pm$ SEM with $n=4$. B) Quantitative measurement of adenylate kinase release in α -syn transfected H4 cells treated with AngII, losartan (L) and/or PD1243319 (P). (*) Shows significance from α -syn alone control, (#) from AngII and (@) from AngII plus losartan or AngII plus PD1243319, with $p < 0.05$ using One-way ANOVA with Newman Keuls post hoc analysis. Data is represented as $\pm$ SEM with $n=4$. C) AngIV (100 nM-1 μ M) in α -syn transfected

cells in the presence of losartan (1 μ M) and PD123319 (1 μ M) (LP). (*) Shows significance from α -syn alone control, (#) from AngIV alone with $p < 0.05$ using One-way ANOVA with Newman Keuls post hoc analysis. Data is represented as + SEM with $n=4$.

Figure 3. AngII mediated reduction of α -syn inclusion formation in transfected H4 cells. A) Representative image of an immunostained cell with or without α -synuclein positive inclusions. B) Quantitative analysis of total α -syn transfected cells with inclusions treated with AngII (100 nM), AngIV (1 μ M), Losartan (L) (1 μ M) and/or PD123319 (P) (1 μ M). (*) Shows significance from α -syn control with $p < 0.05$ using One-way ANOVA with Newman Keuls post hoc analysis. Data is represented as $\pm$ SEM with $n=4$.

Figure 4.

Representative image from immunoblot analysis of α -synuclein (Syn-T) and/or synphilin-1 (V5) transfected H4 cells treated with AngII (100 nM), Losartan (1 μ M) and/or PD123319 (1 μ M). Protein loading was estimated using the BSA method and compared to actin protein bands.