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AMPK over-activation leads to accumulation of α -synuclein oligomers and decrease of neurites

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Abstract

Neuronal inclusions of α -synuclein (α -syn), termed Lewy bodies, are a hallmark of Parkinson disease (PD). Increased α -syn levels can occur in brains of aging human and neurotoxin treated mice. Since previous studies have shown increased brain lactate levels in aging brains, in PD affected subjects when compared to age-matched controls, and in mice treated with MPTP, we tested the effects of lactate exposure on α -syn in a cell based-study. We demonstrated that (i) lactate treatment led to α -syn accumulation and oligomerization in a time- and concentration-dependent manner, (ii) such alterations were mediated via adenosine-monophosphate activated protein kinase (AMPK) and associated with increasing cytoplasmic phosphorylated AMPK levels, (iii) AMPK activation facilitated α -syn accumulation and phosphorylation, (iv) lactate treatment or overexpression of active form of AMPK decreased α -syn turnover and neurite outgrowth and (v) Lewy body-bearing neurons displayed abnormal cytoplasmic distribution of phosphorylated AMPK, which normally is located in nuclei. Together, our results suggest that chronic neuronal accumulation of α -syn induced by lactate-triggered AMPK activation in aging brains may be a novel mechanism underlying α -synucleinopathies in PD and related disorders.

Keywords

Alpha-synuclein; Parkinson's disease; AMPK; lactate

Introduction

Aging and exposure to neurotoxins are considered as the most prominent risk factors for development of idiopathic Parkinson disease (PD) (Andersen et al., 1999; Chu et al., 2002; Calne and Langston, 1983). Ample evidence supported that these two factors can cause mitochondrial dysfunction, which plays a central role in PD pathogenesis (Larsson, 2010; Wang et al., 2011; Zhu and Chu, 2010), and that mitochondria impairment can lead to elevated lactate levels (Weissman et al., 2008; Dykens et al., 2008; Ross et al., 2010). Previous studies have shown increased lactate levels in aging brain (Ross et al., 2010), in PD affected subjects when compared to age-matched controls (Bowen et al., 1995), and in mice

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Disclosure statement

All authors have no actual or potential conflicts of interest. All animal work was approved by the Institutional IACUC.

treated with MPTP (Koga et al., 2006), which is a neurotoxin known to cause PD symptoms and mitochondria dysfunction (Przedborski et al., 2004). Moreover, it has been noted that α -syn levels increase in aging brain (Jellinger, 2004; Li et al., 2004; Chu and Kordower, 2007) as well as in MPTP-treated animals (Vila et al., 2000; Kowall et al., 2000). It remains unknown whether elevation of lactate levels has an impact on α -syn accumulation/aggregation.

Previous studies have demonstrated that lactic acid can upregulate the activity of adenosine monophosphate activated protein kinase (AMPK) (Chen et al., 2010a). AMPK is a serine-threonine kinase protein complex and a central regulator of cellular energy homeostasis (Hardie, 2011). Its activity can also be regulated by physiological stimuli, independent of cell energy charge, including hormones, nutrients (Towler and Hardie, 2007; Chen and Downs, 2008). AMPK consists of α , β and γ subunits. Among them, α subunit displays catalytic activity. Phosphorylation of Thr residue at amino acid #172 in α subunit is essential for AMPK activation to function as a protein kinase (Woods et al., 2000).

Recently, cell-based studies have demonstrated that incubation of cultured cells with lactic acid induces aberrant amyloid precursor protein processing to increase A β 40 and 42 peptides (Xiang et al., 2010) and enhances AMPK activation (Chen et al., 2010a). Besides, H₂O₂ treatment of SH-SY5Y cells was shown to cause not only oxidative stress but also AMPK activation, and such oxidative stress was protected by decreasing AMPK level via gene knockdown (Chen et al., 2010b). Moreover, increased AMPK activation/levels were found in cultured cells treated with MPTP (Choi et al., 2010). In view of these findings and aging being a prominent risk factor for PD, we hereby investigated whether dysregulation of AMPK activation has a role in development of synucleinopathy.

To examine this issue we used experimental models such as differentiated dopaminergic neuroblastoma cells 3D5 (Takahashi et al., 2007) and mouse primary neurons which overexpress wild-type human α -syn by the tetracycline-off (TetOff) inducible mechanism and viral infection, respectively. We upregulated AMPK activity in these cultured cells by exposing the cells to lactate or overexpressing active form of AMPK regarded as AT-AMPK, downregulated AMPK by overexpressing a dominant negative form of AMPK referred to as DN-AMPK (Za'tara et al., 2008), or expressing shRNA of AMPK alpha units (for knockdown) referred to as KD-AMPK. We also determined whether the pattern of activated AMPK distribution in cultured cells and human brain tissues can be affected by lactate treatment and synucleinopathy, respectively.

Materials and Methods

Cell models

Alpha-syn transfectant 3D5 was derived from human neuroblastoma BE2-M17D cell line (Takahashi et al., 2007). 3D5 cells express wild-type human α -syn and display neuronal phenotypes upon TetOff induction and incubation with RA, respectively (Ko et al., 2008). Under certain experimental conditions, 3D5 cells are capable to accumulate oligomeric α -syn and form aggregates. Cultures of 3D5 were maintained in DMEM/10% fetal bovine serum with 2 μ g/mL Tet or without at 37 °C and 5% CO₂. Those intended for biochemical analysis were seeded at 0.5 $\times 10^6$ cells/plate (100 $\times$ 20 mm, BD Biosciences, San Jose, CA) or 1.5 $\times 10^5$ cells/well in 6-well plates. For immunofluorescence or spectrophotometric assay, 3D5 cells were seeded at 2 $\times 10^4$ cells/well on coverslips in 24-well plates and 1 $\times 10^4$ in 48-well plates (Bellco Glass Inc, Vineland, NJ), respectively. Media were replaced next day with Neurobasal medium (Invitrogen, Carlsbad, CA), 2% B-27 supplement (antioxidant free, Invitrogen), 2 mM L-glutamine (Sigma) and 10 μ M RA (Sigma-Aldrich, St Louis,

MO). Cultures were subjected to lactate treatment or lentiviral infection for durations to be described later.

Primary neuronal cultures were prepared from hippocampal and cortical brain tissues. For analyses of neurite outgrowth we used PAC mice (postnatal day 1) with transgenic expression of wild-type human α -syn. The level of α -syn expressed in these mice is no more than 2 fold of that expressed endogenously (Jiang et al., 2010). For studies of α -syn oligomers we used cultures derived from non-transgenic embryonic mice (E17) and infected with lentivirus carrying Myc-tagged human α -syn. This is because of the low expression level of endogenous (mouse) or exogenous (human) α -syn in cultures from PAC mice, hence much more lysates than those from cultures with viral infection are required to detect the signals indicative of α -syn oligomer formations. Experimental protocols for primary cultures were the same as those reported previously (Kivell et al., 2001). Cells were plated at $6\sim7\times10^5$ cells on poly-D-lysine (Sigma) coated 6-well plate for biochemical analysis or $2\sim4\times10^4$ per well on coverslips in 24 well plate for neurite outgrowth analysis. They were infected with lentivirus carrying genes of interest 3~4 days after seeding or exposed to lactate or not after 5~6 days in culture.

Animals

All animal procedures were approved by the Mayo Clinic Institutional Animal Care and Use Committee (IACUC) and were in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23) revised 1996.

Lentiviral plasmids and virus preparation

Lentiviral plasmid carrying Myc tagged wild type α -syn was a gift from Dr. Thomas C. Sudhof (Burré et al., 2010) and those carrying AT-AMPK or DN-AMPK were generously provided by Dr. Rachel Hertz (Za'tara et al., 2008). ShRNAs of human or mouse AMPK alpha units were purchased from Sigma Aldrich. Lentiviruses carrying genes of interest or shRNA were prepared following methods described in the manual of ViraPower Lentiviral Expression Systems from Invitrogen Company. Briefly, lentiviruses were generated by plasmid transfection with helper plasmids in HEK293FT cells. Forty-eight hours after transfection, the media were collected and concentrated using a Fast-Trap Lentivirus Purification and Concentration Kit (Millipore).

Antibodies

Monoclonal antibodies Synuclein-1 (BD Biosciences) and LB509 (Invitrogen) were used to detect total α -syn in cultured cells and tissue specimens, respectively. Antibody pSer129 (Wako Chemicals USA) was for detection α -syn phosphorylated at Ser129. Other antibodies were rabbit polyclonal antibodies to total AMPK (Cell Signaling) or activated AMPK (phospho T172, Abcam) as well as mouse monoclonal antibodies to GAPDH (Covance Research Products), MAP2 (Sigma Aldrich), PARP and HSP90 (Cell Signaling). The binding of primary antibody to protein of interest was detected using peroxidase-conjugated goat anti-rabbit or anti-mouse IgG antibodies (Chemicon), Alexa594, Alexa488 or Cy3 labeled secondary antibodies (Invitrogen).

Western blot analysis

Cell cultures were harvested and centrifuged at $200\times g$ for 15 min. They were lysed in RIPA buffer (Cell signaling) for protein turnover studies or in MES buffer (Jiang et al., 2010) for analysis of AMPK and other proteins. All lysis buffers were supplemented with phosphatase inhibitors. The cell lysates were mixed with Tricine-SDS sample buffer (Invitrogen) and 2% β -mercaptoethanol, boiled for 5 min and resolved by SDS-PAGE using 10–20% Tris/

Tricine gel (Bio-rad, Hercules, CA). Precision plus protein standards (Bio-Rad) were included as references. Proteins separated by SDS-PAGE were transferred onto nitrocellulose and processed for immunolabeling for proteins of interest. Membranes probed with GAPDH antibody were used as loading controls. Immunoreactivity was visualized with enhanced chemiluminescence (ECL plus, Amersham Pharmacia Biotech) or SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Scientific) and analyzed as before (Jiang et al., 2010).

Immunoprecipitation

3D5 cells were infected with lentivirus carrying AT-AMPK and incubated subsequently with RA and concurrently subjected to TetOff induction to express α -syn for 15 days. The experimental paradigm allowed cells to express adequate amounts of AT-AMPK and α -syn. Cell lysates were incubated with Dynabeads® (Invitrogen) coated with rabbit or mouse IgG (served as control), antibodies to p-AMPK or α -syn, following the manufacturer's manual. Proteins bound to the beads were released by boiling in sample loading buffer and probed with antibodies to p-AMPK or α -syn by western blotting.

In vitro phosphorylation assay for α -syn

Human neuroblastoma BE2-M17D cells were infected with lentiviruses carrying AT-AMPK or DN-AMPK, followed by 15 days of RA initiated neuronal differentiation. Cells were harvested, lysed in RIPA buffer and the lysates were incubated with Dynabeads® coated with p-AMPK antibody or rabbit IgG (served as control) at 4°C overnight. The incubated mixtures were isolated and washed to remove proteins not binding to the beads. For *in vitro* phosphorylation assay, some of the beads were incubated with 10 μ g recombinant human α -syn prepared as described previously (Jiang et al., 2008) in a 50 μ l buffer containing 20 mM HEPES, 1 mM $MnCl_2$, 1 mM $MgCl_2$, 1 mM DTT, 100 μ M sodium orthovanadate and 1 mM Na_2ATP for 12 hours (hrs) at 30°C. Some immunoprecipitates of lysates from AT-AMPK overexpressing cultures were incubated similarly in the presence of recombinant α -syn and absence of Na_2ATP to serve as controls. The process of protein phosphorylation was terminated by adding sample loading buffer to the reaction mixtures, followed by boiling. Equal amounts of supernatants from different samples, obtained after removing the beads, were compared by SDS-PAGE and western blotting.

Quantitative real-time PCR

Four sets of 3D5 cultures with RA-elicited neuronal differentiation and TetOff induction were used. They include those (i) maintained for 4 days (Con0) in culture without additional perturbations and (ii) perturbed on the 4th day of culturing with a 6 day exposure to lactate (20 mM, LA6d), (iii) a 6 day infection with lentiviruses containing AT-AMPK (AT6d) or (iv) empty vector (Con6d). Total RNA from each sample was isolated using RNeasy Mini Kit (Qiagen), and 3 μ g were used to synthesize cDNA using SuperScript® III First-Strand Synthesis System (Invitrogen). The reaction mixtures were subjected to quantitative real-time PCR to determine the level of α -syn transcript. Triplicate reactions per sample were prepared using a 25- μ l mixture containing Platinum SYBR Green qPCR Super Mix UDG (Invitrogen). The same analysis was performed with 4 samples from 3 set of experiments. All data were normalized to the endogenous GAPDH expression. The PCR was run on the ABI 7900 and data analyzed using Software RQ Manager 1.2 (Applied Biosystems).

Alpha-syn turnover

To study α -syn protein turnover, we used a strategy in which the expression of α -syn induced by the TetOff mechanism is terminated by adding tetracycline (Tet) back to culture media and followed by determine the level of non degraded α -syn in cultures at different

days after the Tet addition. This approach has been used in previous studies to study protein turnover (Vogiatzi et al, 2008, Hamano et al., 2008). Briefly, 3D5 cultures with RA-elicited neuronal differentiation and TetOff induction for 4 days were perturbed with lactate treatment (LA), infected with lentiviruses carrying AT-AMPK (also regarded as AT) or empty vector (Con). Three days later, Tet (2 μ g/ml) was added back to culture media to turn off the expression of exogenous α -syn. For monitoring the turnover of α -syn, subsets of cells from each group were harvested at 0, 2 and 4 days after the addition of Tet. RIPA buffer (Cell Signaling) was used for protein extraction to dissociate oligomeric α -syn. The cell lysates obtained were probed with antibodies to α -syn, and GAPDH by western blotting.

Immunocytochemistry

Cells grown on coverslips were rinsed with PBS, fixed in 4% paraformaldehyde and permeabilized with 0.1 M Tris-buffered saline (TBS, pH 7.6) containing 0.5% triton X-100 for 5 minutes (mins). They were subsequently blocked with 3% goat serum in TBS, incubated with antibody Synuclein-1 and p-AMPK in TBS containing 1% goat serum overnight at 4 °C then incubated for 1 hour with goat anti-rabbit antibody conjugated with Alexa594 or goat anti-mouse conjugated with Cy3. Immunolabeled cells were stained with nuclei stain DAPI (Invitrogen) for 10 mins and evaluated by confocal fluorescence microscopy (Zeiss LSM 510, Carl Zeiss MicroImaging).

Immunohistochemistry

Sections of paraffin embedded tissue were cut at 5 microns thickness, allowed to dry overnight at 60°C and followed by deparaffinization and rehydration. For immunoperoxidase labeling, tissue sections were steamed for 30 mins in Dako target retrieval solution, pH 6.1. Following antigen retrieval and blocking of endogenous peroxidase with 3% hydrogen peroxide, the sections were incubated with 5% normal goat serum for 20 min to reduce non-specific labeling. Tissue sections were then incubated with anti-p-AMPK (1:750) for 45 mins and Envision-Plus labeled polymer HRP, rabbit (Dako) for 30 mins. Peroxidase labeling was visualized with a solution containing 3, 3'-diaminobenzidine (DAB-Plus). The sections were subsequently counterstained with Lerner 1 hematoxylin (14-930-11, Fisher Scientific) and coverslipped with cyto seal mounting medium (8310-16, Richard-Allan Scientific). For immunofluorescence, sections were incubated with 0.1 mg/ml of protease XXIV (P8038, Sigma Aldrich) for 8 mins, steamed for 30 mins in dH₂O, rinsed and incubated with Protein Block (X0909, Dako) for 1 h at room temperature. These sections were then incubated with LB509 (anti- α -syn, 1:50) and ab51110 (anti-p-AMPK, 1:100, Abcam) at 4°C overnight, followed by 1.5 hs incubation with secondary antibodies (1:500) after washing. Non-specific fluorescence signals were blocked by staining with Sudan Black stain for 2 mins. Sections were coverslipped with Vectashield mounting media (H-1200, Vector Laboratories) and evaluated by confocal fluorescence microscopy.

Neurite outgrowth analysis

Embryonic cortical/hippocampal neurons from PAC α -syn mice were cultured for 3 days, and then infected with lentivirus carrying genes or shRNAs of interests. After a 2-d infection we added lactate to culture media and processed the cells for MAP2 immunolabeling 12 days later. The impacts of lactate treatment on outgrowth of neurites in 30 or more neurons from 3 independent cultures were analyzed using the METAMORPH software (Universal Imaging Corporation, Downingtown, PA, USA).

Statistical analysis

Data from at least 3 sets of independent experiments were analyzed by one-way Anova with Dunnett's *post hoc* test to determine statistical significance.

RESULTS

Exposure to lactate causes accumulation of α -syn in 3D5 cells in a time- and concentration-dependent manner

To determine the effects of lactate exposure on α -syn accumulation, we treated 3D5 cultures with 20 mM sodium lactate (referred to as lactate or LA) for 3 or 6 days or with different concentrations (0, 4, 8, 16, 24 mM) of lactate for 3 days. Prior to the treatment, 3D5 cultures were differentiated with retinoic acid (RA) and induced to express α -syn for 4 days. Sibling cultures without lactate treatment were included as controls. As demonstrated in previous studies (Ko et al., 2008; Jiang et al., 2010), non-treated controls were capable to accumulate multiple α -syn species detectable with antibody Synuclein 1 (Fig. 1A). The variety and amount of α -syn species increased as the duration of TetOff induction extended (compared Con3d to Con 6d, Figs. 1A and 1C). Significant enhancement of such accumulation of different α -syn species were detected in cultures exposed to 20 mM lactate and more in those with longer exposure (Figs. 1A and 1C), indicating α -syn accumulation is a time-dependent process. Alpha-syn accumulation/oligomerization was not easily detected in cultures with less than 2 days of lactate treatment, indicating chronic (days as opposed to minutes) lactate exposure is necessary. Our analyses of cultures treated with different concentrations of lactate demonstrated further that α -syn accumulation induced by lactate is concentration-dependent (Figs. 1B and 1C).

Chronic exposure of lactate causes α -syn accumulation in primary neuronal cultures overexpressing human α -syn

To determine whether the effects of lactate treatment on α -syn accumulation are unique to 3D5 cultures, we included primary neuronal cultures derived from embryonic brains of non-transgenic mice. These cultures were infected with lentivirus carrying Myc-tagged human α -syn for 2 days, followed by exposure to 20 mM lactate for 6 and 12 days. Consistent with what we observed in 3D5 cultures, lactate treatment led to significant increase of α -syn (total) accumulation when compared to non-treated control (Fig. 2, e.g. compared LA6d to Con6d). The profile of different sizes α -syn detected in primary cultures, however, differed from those accumulated in 3D5 cells. This may reflect intrinsic differences between human and mouse cells, differences in α -syn expression levels, culture conditions or other unknown factors.

Lactate treatment elicits AMPK activation in neuronal cultures

To identify mechanisms underlying lactate-induced α -syn accumulation, we analyzed the level of AMPK which is known to involve in signaling pathways sensitive to alteration of lactate levels (Chen et al., 2010a). After normalizing the immunoreactivity of phosphorylated AMPK (p-AMPK) with that of total AMPK (T-AMPK) or GAPDH, we found that the level of activated AMPK was significantly increased in cultures with 3 or 6 days of 20 mM lactate exposure (Figs. 1A and 1D). Importantly, the extent of AMPK activation was greater in cells with higher doses of lactate (Fig. 1D), suggesting a link between AMPK activation and increased accumulation of different α -syn species in the lactate-exposed.

Enhanced AMPK activity facilitates α -syn accumulation and phosphorylation in neuronal cultures

To assess the role of AMPK activation in α -syn accumulation, we infected RA-differentiated, α -syn overexpressing 3D5 cells and primary neurons with different doses of lentivirus carrying AT-AMPK or empty vectors as control. AT-AMPK contains only the first 312 amino acid residues (catalytic domain) of AMPK α subunit, hence expression of this domain leads to production of constitutively active AMPK (Foretz et al., 2005; Za'tara et al., 2008; Crute et al., 1998). Lysates from 3D5 cells and primary neuronal cultures collected 6 and 12 days post infection, respectively, were analyzed for levels of total and phosphorylated α -syn, phosphorylated AMPK and GAPDH. As expected, cultures infected with AT-AMPK, but not empty vectors, expressed proteins of size comparable to that of AT-AMPK (~ 36kDa). In comparison to controls, more α -syn proteins were accumulated in cultures expressing AT-AMPK, and the differences were greater in cultures with its higher doses of viral infection (Compared 1xAT to 2xAT in Figs. 3A and 3C). We also noted that AT-AMPK expression results in increasing the ratio of phosphorylated to total α -syn (Figs. 3A and 3D), suggesting α -syn may be a substrate of activated AMPK.

Reducing AMPK activity inhibits α -syn accumulation caused by lactate treatment

AMPK activity can be down regulated by overexpressing its shRNA for knockdown, or by overexpressing its dominant negative form (DN), which contains a mutation that alters aspartic acid residue 157 to alanine thus competes with its native α subunit for binding with β and γ subunits to inhibit AMPK activation (Woods et al., 2000; Za'tara et al., 2008). To confirm further the role of activated AMPK in α -syn accumulation, we used 3D5 cells and primary neuronal cultures with reduced AMPK activity. These were achieved by infection of cultures with lentivirus carrying shRNA of AMPK or DN-AMPK (Fig. 4). With GAPDH normalization we found that 3D5 and primary cultures expressing shRNA of AMPK contain significant lower levels of T-AMPK than those without the shRNA, and that cultures expressing DN-AMPK contain more T-AMPK as expected (Figs. 4A and 4B). Both AMPK knockdown and DN-AMPK expression caused a significant reduction of active AMPK (i.e. decrease of p-AMPK; Figs. 4A and 4C). Regardless of difference in cell origin, cells with reduced AMPK activity were more resistant to α -syn accumulation and phosphorylation caused by lactate exposure when compared to empty vector infected counterparts (Figs. 4A–4E). The level of monomeric α -syn increased in cultures treated with lactate, as shown in western blots with optimum exposures (Fig. 4A). Such effect was not detected in lactate-treated cultures with AMPK knockdown or DN-AMPK overexpression. It is worth mentioning that detection of oligomeric α -syn requires longer exposure of western blots (e.g. Figs. 1 and 2).

Immunoprecipitation demonstrates interactions between AMPK and α -syn

The issue of whether α -syn is a substrate of AMPK was investigated using immunoprecipitation. Immunoprecipitates (IP) obtained from incubating lysates of 3D5 cell overexpressing α -syn and AT-AMPK with antibodies to α -syn, p-AMPK, rabbit or mouse IgG were probed using antibodies to p-AMPK and α -syn (Fig. 5). As expected, IP/ α -syn and IP/p-AMPK contained α -syn and pAMPK (Figs. 5A and 5D) respectively, plus immunoglobulins (light and heavy chains, marked by asterisk). IP of α -syn (IP/ α -syn) from the aforementioned cultures displayed two p-AMPK immunoreactive bands of molecular size similar to that of full-length AMPK (~ 62 KDa) and AT-AMPK (~36 kDa) shown in the lysate prior to immunoprecipitation (Fig. 5B). Both AMPK products were not detected in the IP obtained from incubation of cell lysates with mouse IgG. IP/p-AMPK from AT-AMPK overexpressing cells contained α -syn immunoreactive proteins of size comparable to that of α -syn monomer (~15 kDa) and dimer (~30 kDa) shown in the lysate before immunoprecipitation (Fig. 5C). Such α -syn products were not detectable in the precipitates

from rabbit IgG incubated controls (IP/IgG). Together the results support the notion that AMPK and α -syn can interact with each other either directly or indirectly via other proteins pulled down specifically by antibodies to p-AMPK and α -syn.

Alpha-syn is a substrate of activated AMPK

To determine whether interactions between α -syn and p-AMPK lead to α -syn phosphorylation, we performed *in vitro* phosphorylation assays of recombinant α -syn in four reaction mixtures containing IP/p-AMPK or IP/IgG of lysates from AT-AMPK or DN-AMPK expressing cultures (see Fig. 6 for details). Na₂ATP was excluded in one of the four mixtures to serve as control, and IP/IgG were used to assess the specificity of IP/ p-AMPK. After incubation, all mixtures were analyzed for the level of total and phosphorylated α -syn as described earlier. Western blotting demonstrated that reaction mixtures containing ATP plus IP/p-AMPK of lysates from cell overexpressing AT-AMPK have a significantly higher level of phosphorylated α -syn than those from cultures expressing DN-AMPK (Figs. 6A and 6B), or from those containing IP/IgG. Reaction mixtures containing IP/-AMPK of lysates from DN-AMPK cultures displayed some signals of α -syn phosphorylation. This is due to presence of residual p-AMPK in these cultures (see Fig. 4). Phosphorylated α -syn was not detectable in the reactions mixture lacking ATP. Together, the results support the notion that α -syn is a substrate of AMPK.

Chronic Lactate treatment significantly reduces neurite outgrowth and reducing AMPK activity prevents the change

To study the impacts of lactate treatment on neurons we measured the total length of neurites extended from neurons in primary cultures, since loss of neurites can result in synaptic dysfunction to cause neuronal degeneration (Chernova et al., 2007; Loh et al., 2008). Our measurement of multiple neurons showed a significant reduction in the total length of MAP2-positive neurites in neurons from PAC mice by lactate treatment or AT-AMPK overexpression when compared to controls (Fig. 7). These experimental perturbations did not affect cell viability. Similar reduction of neurite outgrowth was not detected in lactate-treated cultures expressing shRNA of AMPK or DN-AMPK (Fig. 7). We noted that lactate treatment of cultures with active AMPK (AT-AMPK) plus α -synuclein overexpression not only reduced the length of neurites further, but also decreased cell viability. Neurite outgrowth and cell viability of cultures without α -synuclein overexpression were not affected by lactate exposure (Fig. 7, nontransgenic), suggesting that the vulnerability of neurons to lactate treatment can be altered by low levels of expressing exogenous human wild-type α -syn.

Lactate treatment or AT-AMPK overexpression decreases α -syn turnover in 3D5 cells without affecting its transcription

We next determine if AMPK activation-induced α -syn accumulation was caused by altering its transcript levels or turnover. We analyzed 3D5 cultures with lactate exposure, AT-AMPK overexpression or without (control). The results (Fig. 8A) of quantitative real-time PCR analyses showed that 6 days of lactate treatment (LA6d) or AT-AMPK overexpression (AT6d) did not have a significant effect on the level of α -syn transcripts when compared to controls cultures infected with empty vector (Con6d) and maintained for the same durations. In contrast, higher levels of α -syn transcript were detected in cultures with longer duration of α -syn induction as expected (compared Con6d with Con0d, which denotes samples harvested after 10 and 4 days of Tetoff induction).

For monitoring α -syn protein turnover, we compared lysates collected from cultures after 0, 2 and 4 days of adding back Tet to culture media, since the addition inhibits induced protein expression and α -syn has a relatively long half-life (Okochi et al., 2000; Sevlever et al.,

2008). Our quantitative analyses of immunoblots with GAPDH normalization showed that following the addition of Tet, higher levels of non degraded α -syn were detected in the lactate-treated or AT-AMPK overexpressed cultures than corresponding controls. The differences are statistically significant (Figs. 8B and 8C).

Lactate treatment increases the level of activated AMPK in cytoplasm

Previous studies of many cell types have shown that under normal condition activated AMPK is predominantly distributed in nucleus (Vingtdeux et al., 2011), whereas α -syn is mostly located in cytoplasm. Therefore, the enhanced α -syn phosphorylation demonstrated in our lysates from lactate-treated cells could result from increasing the level of active AMPK in cytoplasm. To address this issue, RA-elicited differentiated 3D5 cells with 4 days of induced α -syn expression were treated with or without lactate for 6 days and used for immunocytochemical and cell fractionation and analyses. By immunofluorescence labeling of non-treated controls we showed the predominant distribution of α -syn and p-AMPK in cytoplasm and nucleus, respectively, as expected (Con in Fig. 9A). In comparison to controls, increased signals of cytoplasmic p-AMPK immunolabeling in a punctate pattern were detected in lactate-treated cultures (LA in Fig. 9A). The lactate treatment had no apparent effects on α -syn distribution. Dual immunolabeling showed co-localization of α -syn and p-AMPK in the cytoplasm of the lactate-treated (Fig. 9A). By immunoblotting and using PARP and HSP60 immunoreactivities as references for samples from nuclear and cytoplasmic fractions, respectively, we showed that lactate treatment causes a significant increase of p-AMPK levels in cytoplasmic fraction without affecting the level of total cytoplasmic AMPK. The treatment also led to an increase of total α -syn in the cytoplasmic fraction without much effect on the level of nuclear α -syn (Figs. 9B – 9E). The results are consistent with those demonstrated by immunocytochemistry.

Detection of activated AMPK in cytoplasm of human neurons bearing α -syn pathology

To determine whether synucleinopathy associates with abnormal distribution of activated AMPK, we performed immunohistochemical studies of 12 human brains obtained at autopsy. Half of them were from subjects affected with Diffuse Lewy body disease (DLBD) and half were age-matched controls. Upon immunoperoxidase labeling with antibodies to p-AMPK, we detected p-AMPK immunoreactivity in some neurons in DLBD specimens (Fig. 10A) and showed these neurons containing Lewy bodies. Dual immunofluorescent labeling showed staining of p-AMPK not only at/or near the rim of Lewy bodies (α -syn positive) but also at the rest of cytoplasm. In contrast, specimens from normal control did not display cytoplasmic p-AMPK immunolabeling, but showed staining of nuclei.

Discussion

Previous genetic studies have shown that some patients with familial PD overexpress wild-type α -syn due to multiplications of its gene *SNCA* and that the clinical phenotype correlates with such upregulation (Singleton and Gwinn-Hardy, 2004; Singleton et al., 2003; Chartier-Harlin et al., 2004; Nishioka et al., 2006). Moreover, the risk to develop PD increases with polymorphisms within the *SNCA* promoter (Pals et al., 2004; Hadjigeorgiou et al., 2006; Chiba-Falek et al., 2005). These findings, along with α -syn levels increase associated with aging (Jellinger, 2004; Li et al., 2004; Chu and Kordower, 2007), a prominent risk pertinent to the onset of PD, suggest that accumulation of α -syn heightens vulnerability to the development of neurodegeneration in PD. The mechanisms underlying how α -syn accumulates and forms filamentous inclusions in PD and related synucleinopathies remain elusive.

In considering the findings of increased lactate levels and impaired energy metabolism in aging brains (Roberts and Rosenberg, 2006), we tested whether exposure to lactate has any impact on α -syn accumulation (monomers plus oligomers). Using the TetOff-induction or lentiviral-transduction mechanism to increase α -syn expression, we demonstrated the accumulation of increased amounts of monomeric, different sized oligomeric and phosphorylated α -syn in neuronal cultures in a time- and dose-dependent manner upon exposure to lactate.

To understand how lactate exposure changes α -syn accumulation and phosphorylation, we tested whether the effects can be blocked or enhanced by manipulating the levels of some other proteins sensitive to the exposure. One of them is a protein kinase named AMPK, which was shown in current and previous studies (Chen et al., 2010a) to be more abundant/active in cultures treated with lactate than those without. In addition, such change of AMPK was accompanied by increase of α -syn in terms of quantity, variety of species and extent of phosphorylation. The link between these changes and AMPK activation was supported by our data obtained from studies of cultures overexpressing active form of AMPK, in which greater changes of α -syn accumulation were found in cultures expressing higher level of active form of AMPK. Moreover, cultures expressing a dominant negative form of AMPK or with AMPK knockdown were less responsive to lactate-elicited α -syn accumulation and phosphorylation. These findings suggest a role for activated AMPK in mediating changes of α -syn properties caused by lactate exposure. The increase of oligomeric α -syn observed in lactate-treated or AT-AMPK expressed cultures may be a consequence of increasing the amounts of monomeric α -syn for AMPK mediated phosphorylation and self-interactions, since these perturbations did not affect the level of α -syn transcript, but reduce α -syn protein turnover.

The possibility of AMPK as a kinase for α -syn phosphorylation was supported further by our analyses of immunoprecipitates and *in vitro* phosphorylation assays. Such studies demonstrated co-immunoprecipitation of α -syn and activated AMPK, hence support interactions between these molecules in cell lysates either directly or indirectly. It has been well documented that in human synucleinopathies (Fujiwara et al., 2002; Saito et al., 2003) and PD animal models (Wakamatsu et al., 2007; Takahashi et al., 2003; Yamada et al., 2004) α -syn in inclusions is phosphorylated at S129. Moreover, *in vitro* studies have shown phosphorylation enhancing α -syn's susceptibility to form aggregates (Fujiwara et al., 2002). Although, it remains debatable as to whether phosphorylation affects α -syn assembly *in situ*/*in vivo*, our studies clearly showed a close link between the level of α -syn accumulation/phosphorylation and the extent of AMPK activation/expression.

Interactions between molecules are less likely to occur if they are far apart from each other. In this regard our immunofluorescence studies of cultures without lactate exposure showed none or very little co-distribution of activated AMPK and α -syn, with one in nucleus and the other in cytoplasm, indicating that under normal condition α -syn may not be phosphorylated by activated AMPK. However, in cultures treated with lactate, some activated AMPK was located in the cytoplasm and co-distributed with α -syn. Such change of p-AMPK distribution was demonstrated clearly by comparing immunoblots of cytoplasmic and nuclear fractions derived from cultures with and without lactate treatment. The change was accompanied with increased levels of α -syn phosphorylation in cytoplasmic fraction. These findings as well as those described earlier provide strong supports for a role of AMPK in α -syn phosphorylation. Our studies showed that lactate treatment or expressing active AMPK does not affect α -syn transcription, but leads to decrease of α -syn turnover. In this regard it has been reported that aging brains contained increased levels of α -Syn and lactate (Jellinger, 2004; Li et al., 2004; Chu and Kordower, 2007; Ross et al., 2010). Therefore, it is

important in further studies to identify protein degradation pathways affected by lactate/AMPK activation.

As revealed from numerous previous studies, AMPK activation can be linked to neuronal survival in response to different types of stress (e.g. glucose depletion, glutamate exposure, calcium overloading) (Culmsee et al., 2001; Weisová et al., 2011; Weisova et al., 2009; Salminen et al., 2011). However, there are also abundant evidences indicating that AMPK activation can have detrimental impacts (Chen et al., 2010b; McCullough et al., 2005; Li and McCullough, 2010; Li et al., 2007). A recent study of experimental models of motor neuron disease has demonstrated that expression of mutated SOD1 caused increase of AMPK activation, and that reduction of AMPK activity protected these models from motor impairment (Lim et al., 2012). Such dual function of AMPK probably reflects different extents of its activation resulted from different durations or doses of exposure to insults (Salminen et al., 2011). For example, one report showed that modest AMPK activation induces neurogenesis and improves cognition while augmented AMPK activity reduces cognition and catecholamine levels as well as increases neural apoptosis and mortality (Dagon et al., 2005); another report showed that extended AMPK activation can induce expression of Bim, which is a member of the proapoptotic Bcl-2 family (Concannon et al., 2010). Our studies demonstrated that AMPK activation via chronic lactate treatment or overexpressing AT-AMPK had very little effects on cell survival, but significantly reduced the total length of neurites. It is possible that neuritic changes caused by chronic lactate exposure in aging brains may reduce neuronal functions and result in neurodegeneration.

Does AMPK activation play a role in synucleinopathies? Our studies of human specimens showed differences between LB-bearing neurons and normal counterparts in distribution of activated AMPK. Activated AMPK in LB-bearing neurons was located at or near the rim of LB as well as in other areas of cytoplasm. The shift of activated AMPK location from nuclear to cytoplasm in human specimens appeared to be more drastic than what was observed in cultures with lactate treatment. This may reflect differences in durations of exposure to insults, since it takes years to develop synucleinopathies in PD and related disorders.

Abnormal distribution and activation of AMPK are not unique to disorders with synucleinopathy. In brain specimens from cases with Alzheimer's disease, neurofibrillary tangles were immunoreactive with antibodies to phosphorylated AMPK (Vingtdeux et al., 2011). Other studies have shown that AMPK signaling can regulate amyloidogenesis (Won et al., 2010). Thus, our findings and those of others support an important role for AMPK signaling pathways in neurodegenerative diseases.

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Figure 1. Chronic lactate treatment of 3D5 cells induces AMPK activation and α -syn accumulation in a time- and dose-dependent manner

Cells with retinoic acid elicited neuronal differentiation and 4 days of TetOff induction were treated with (A) 20 mM lactate (LA) for 3 and 6 days or (B) 0 mM, 4 mM, 8 mM, 16 mM and 24 mM lactate for 3 days. Cell lysates were probed with antibodies to α -syn, phosphorylated AMPK (p-AMPK), total-AMPK (T-AMPK) and GAPDH by western blotting. Molecular weight standards and lysates from cells without exposing to lactate were included as references. The results of quantification of α -syn/GAPDH and p-AMPK/T-AMPK immunoreactivities demonstrated in (A), (B) and immunoblots from additional studies were shown in (C) and (D) as percentage of non-treated controls group [i.e. Con0d for (A) and 0 mM for (B)]. In non-treated controls more different sizes α -syn were significantly accumulated in those with longer duration of α -syn overexpression. Lactate treatment caused a significant increase of total α -syn accumulation in a time and concentration manner. Error bars represent standard error of the mean (SEM) (* $p < 0.05$, ** $P < 0.01$, $n = 3$). The results are representative of 3 independent experiments.

Figure 2. Chronic lactate treatment leads to AMPK activation and α -syn accumulation in primary neuronal cultures

Lysates from neuronal cultures were probed with antibodies to α -syn, p-AMPK, T-AMPK and GAPDH. The cultures were derived from embryonic (E17) mouse brains, infected with lentivirus carrying Myc-tagged human α -syn for 4 days and followed by 6 and 12 days of exposure to 20 mM lactate, referred to as LA6d and LA12d, respectively. Duplicate cultures with viral infection, but without lactate treatment for 0, 6 and 12 days were included as controls and marked as Con0d, Con6d and Con12d, respectively. Quantitative analyses of immunoreactivities in (A) and immunoblots from additional experiments were performed and the ratio of α -syn/GAPDH and p-AMPK/T-AMPK immunoreactivity were shown in (B) and (C). Bar graphs showed the values of control groups (Con0d) as 100%. Lactate treatment significantly increased the level of total α -syn and that of active AMPK (i.e. p-AMPK). Error bars represent SEM (* p <0.05, ** p <0.01, n =3). The results are representative of 3 independent experiments.

Figure 3. Overexpression of AT-AMPK significantly increases AMPK activity (T172 phosphorylated), α -syn accumulation and phosphorylation in 3D5 cells and primary neurons overexpressing human α -syn

3D5 cells and primary neuronal cultures overexpressing α -syn without and with Myc tag, respectively, were infected with 1 arbitrary unit (1x) or 2 units (2x) of lentivirus carrying AT-AMPK and harvested after a 6 days (3D5 cells) and 12 days (primary cultures) of infection. Cells infected with lentivirus carrying empty vector were used as control (Con). Cell lysates were probed with antibodies to total (Synuclein 1) and phosphorylated (p-S129) α -syn, p-AMPK, T-AMPK and GAPDH via western blotting. Results of quantifications of p-AMPK/GAPDH, α -syn/GAPDH and p-syn/ α -syn immunoreactivities in (A) and

additional experiments were shown in (B) to (D). Bar graphs showed the values of control groups (Con) as 100%. Cultures overexpressing AT-AMPK contained significantly higher levels of total α -syn and phosphorylated α -syn as well as p-AMPK. These changes are greater in those with its higher doses of viral infection. Error bars represent SEM (* $p < 0.05$, ** $P < 0.01$, $n = 3$). The results are representative of 3 independent experiments.

Figure 4. Suppression of AMPK activation inhibits α -syn accumulation and phosphorylation in neuronal cells caused by lactate exposure
 (A) Left panel: 3D5 cells differentiated with RA and induced α -syn expression; Right panel: Primary neuronal cultures expressing Myc-tagged human α -syn. The cells were infected with lentivirus carrying empty vector (Con), DN-AMPK (DN) or shRNAs of human or mouse AMPK alpha units (KD) as described in the Method section. Two days post infection 3D5 cells and primary cultures were treated for 3 and 6 days, respectively, with or without 20 mM lactate treatment. Corresponding samples with lactate treatment were referred to as LA, DN/LA and KD/LA, respectively. Cell lysates were probed with antibodies to total and phosphorylated α -syn (p-syn), total (T) and phosphorylated AMPK (p-AMPK) and GAPDH. Results of quantifying the immunoreactivities of T-AMPK/GAPDH, p-AMPK/GAPDH, total α -syn/GAPDH and p-syn/ α -syn in (A) and additional experiments were

shown in (B) to (E). Bar graphs showed the values of control groups (Con) as 100%. The level of monomeric, total and phosphorylated α -syn as well as that of p-AMPK increased significantly by in LA-treated cultures, but not in those with DN/LA or KD/LA (* $p < 0.05$, ** $P < 0.01$, $n = 3$). As expected the level of T-AMPK was significantly less in KD and more in DN cultures than controls. Error bars represent SEM. Asterisk(s) right above the error bar indicated a significant difference between this group and corresponding control (Con). The results are representative of 3 independent experiments.

Figure 5. Interactions between activated AMPK and α -syn

Lysates of cells overexpressing α -syn plus active AMPK (AT-AMPK) were aliquoted. Some were incubated with antibodies to α -syn or p-AMPK to generate immunoprecipitates, regarded as IP/p-syn and IP/p-AMPK, respectively. To verify the specificity of IP some lysates were incubated with mouse or rabbit IgG, and the precipitates obtained were referred to as IP/m IgG and IP/r IgG, respectively. Cell lysates lacking the aforementioned incubation were also included as references. A portion of each aforementioned sample was probed with antibodies to α -syn or p-AMPK to confirm the enrichment of these proteins in IP (A & D), and evaluate their interactions (B & C). Western blotting of IP/ α -syn with anti-p-AMPK showed two sizes of p-AMPK immunoreactive proteins of sizes consistent with full-length AMPK (~62 kD) and AT-AMPK (~36 kD) detected in cell lysates. IP/p-AMPK was shown to contain different sizes of α -syn positive proteins corresponding to monomer, dimer and larger oligomer. Arrow heads denote activated endogenous and exogenous AMPK. Arrows denotes both monomeric and dimeric α -syn. Asterisk (*) denotes the heavy chain (55kd) and light chain (25 kd) of IgG in IP. The results are representative of 3 independent experiments.

Figure 6. Activated AMPK phosphorylates α -syn *in vitro*

Same amount of recombinant α -syn was used for phosphorylation assays of 4 different mixtures, which contain IP/p-AMPK of lysates from BE2-M17D cells expressing (Exp) AT-AMPK (AT) or DN-AMPK (DN), or IP/IgG of lysates from BE2-M17D cells expressing AT-AMPK. ATP was not included in one of the reaction mixtures to serve as a control.

Upon the phosphorylation assays were terminated, an equivalent amount of different reaction mixtures was probed for total (top panel in A) and phosphorylated (bottom panel in A) α -syn using antibodies Synuclein 1 and p-S129, respectively. A representative of 3 independent experiments was shown in (A). (B) Quantitative analyses showed the level of α -syn phosphorylation, as evaluated by the ratio of phosphorylated α -syn to total α -syn, differed significantly among different preparations (** $P < 0.01$, $n = 3$). Samples containing ATP plus IP/p-AMPK from lysates of AT-AMPK expressing cultures displayed much more phosphorylated α -syn than corresponding IP from lysates of DN-AMPK or IgG precipitates.

Phosphorylated α -syn was not readily detected in samples lacking ATP. Error bars represent SEM. Asterisk(s) right above the error bar indicated a significant difference between this group and group 3. The results are representative of 3 independent experiments.

Figure 7. Lactate treatment or overexpressing AT-AMPK causes reduction of neurite outgrowth and suppression of AMPK expression or activation is protective

Representative micrographs showed images of neurons in primary neuronal cultures from PAC wild type α -syn transgenic mice and non-transgenic littermates under different experimental conditions. These include neurons infected with lentivirus carrying (i) empty vector (control), (ii) AT-AMPK (AT), (iii) empty vector plus treatment with 20 mM lactate (LA), (iv) DN-AMPK plus treatment with 20 mM lactate (DN/LA) or (v) shRNA of mouse AMPK alpha units plus exposure to 20 mM lactate (KD/LA). Scale bar: 50 μ m. Bar graph shows data summarized from quantitative and statistical analyses of total length of neurites per neuron examined. Error bars represent SEM (* $p < 0.05$). The results are representative of 3 independent experiments.

Figure 8. Lactate treatment or AT-AMPK overexpression decreases α -syn turnover in 3D5 cells without affecting its transcription

(A). Quantitative real-time PCR showed no significant differences ($p > 0.05$, $n=3$) in the level of α -syn transcripts among 3D5 cells perturbed for 6 days with lactate treatment (LA6d), lentiviral infection of AT-AMPK (AT6d) and lentiviral infection of empty vector (Con6d). Such perturbation was initiated on day 4 of RA-differentiation and TetOff induction, and cells were harvested on day 4 for Con0d and day 10 for the rest. The level of α -syn transcript in non-perturbed cells was significantly ($p < 0.01$, $n=3$) higher in those harvested with 10 than 4 days of differentiation and induction (Con6d vs Con0d) as expected. LA treatment or expressing AT-AMPK (AT) had no significant effects on the level of α -syn transcript. (B) 3D5 cultures with 4 days of TetOff induction were perturbed with or without lactate treatment (LA), infected with lentiviruses carrying AT-AMPK (also regarded as AT) or empty vector (Con). Three days later, Tet ($2\mu\text{g/ml}$) was added back to culture media to turn off the expression of exogenous α -syn, and cultures were harvested after 0, 2 and 4 days of the Tet addition. The addition of Tet is known to turn off induced α -syn expression. The lysates of Tet added cultures were probed with antibodies to α -syn and GAPDH as shown in (B). Asterisk (*) indicates a significant difference between marked groups and control. Error bars represent SEM. Analysis of scatter plots between non-degraded α -syn (normalized with GAPDH) levels in different samples and duration of Tet addition was shown in (C). The level of non-degraded α -syn (normalized with GAPDH) in cultures harvested at day 0 was regarded as 100%. The slope (mean $\pm$ SEM) of extrapolation line obtained from different samples was presented. Statistical analysis showed a significant ($*p < 0.05$, $n=3$) decrease of α -syn degradation by LA-treatment or AT-AMPK expression when compared to control.

Figure 9. Lactate treatment increases cytoplasmic distribution of activated AMPK in neuronal cells

3D5 cells were induced to express α -syn for 10 days under RA initiated differentiation. On day 4 they were treated with or without 20 mM lactate and referred to as LA or Con, respectively. They were used for dual immunofluorescence labeling with antibodies to p-AMPK and α -syn, followed by staining with DAPI. Panel (A) showed increase of p-AMPK immunolabeling in cytoplasm of the LA-treated. The treatment has very little effects on α -syn distribution. scale bar: 10 μ m. Panel (B) showed western blotting of cytoplasmic and nuclear fractions from LA-treated and non-treated controls, using antibodies to p-AMPK, T-AMPK, α -Syn, PARP and HSP90. PARP and HSP90 immunoreactivities were used to check the purity of nuclear and cytoplasm fraction, respectively. Panels (C) to (E) showed the results of quantitative and statistical analyses of data from 3 independent experiments. Cytoplasmic fraction from lactate treated cultures has a significantly higher ratio of p-AMPK to T-AMPK or α -syn to HSP60 (** $P < 0.01$, $n = 3$). In contrast, LA treatment had no significant effects on the ratio of p-AMPK to T-AMPK or α -syn to PARP in nuclear fraction, or the level of T-AMPK to PARP (nuclear) or HSP90 (cytoplasm). Error bars represent SEM. Asterisk (s) right above the error bar indicated a significant difference between this group and its corresponding control (Con). The results are representative of 3 independent experiments.

Figure 10. Subcellular distribution of activated AMPK differs between neurons with and without bearing Lewy bodies

Human specimens containing Basal nucleus of Meynert from different cases with Diffuse Lewy body disease (DLBD) were probed with antibodies to p-AMPK and α -syn by immunocytochemistry and immunofluorescence. In LB-bearing neurons, p-AMPK immunoreactivities were located at the rim of LBs (Left panel in A & upper panel in B) and the rest of neuronal cell bodies. In neurons from basal nucleus of age-matched normal controls, p-AMPK immunoreactivities located mainly in nuclei (Right panel in A & lower panel in B). Arrow head or red label denotes LBs; arrows or green label denotes p-AMPK immunolabeled structures. Scale bar: 20 μ m in A and 2 μ m in B.