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Author/Contributor:

Dzamko, Nicolas; Chua, Germaine; Ranola, Madelaine; Rowe, Dominic B; Halliday, Glenda

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Measurement of LRRK2 and Ser910/935 phosphorylated LRRK2 in peripheral blood mononuclear cells from idiopathic Parkinson's disease patients

Nicolas Dzamko^{a#}, Germaine Chua^a, Madelaine Ranola^b, Dominic B. Rowe^b and Glenda M. Halliday^{a#}

^a School of Medical Sciences, University of NSW and Neuroscience Research Australia, Sydney, Australia. ^b Australian School of Advanced Medicine, Macquarie University, Sydney, Australia.

Running title: Peripheral blood LRRK2 in Parkinson's disease

Correspondence to n.dzamko@neura.edu.au or g.halliday@neura.edu.au

Abstract

A significant number of autosomal dominantly inherited Parkinson's disease (PD) cases are due to mutations in the leucine-rich repeat kinase 2 (LRRK2) gene. In cells, these pathogenic mutations have a number of differing effects on LRRK2 enzymatic activity and protein stability. In particular, five of the six described pathogenic LRRK2 mutations ablate the constitutive phosphorylation of LRRK2 on Ser910 and Ser935, two residues required for binding of LRRK2 to 14-3-3 proteins. This suggests a potential pathogenic role for these residues. However, LRRK2 kinase inhibitors, which have shown early promise as neuroprotective agents, also ablate the phosphorylation of Ser910 and Ser935. Additionally, LRRK2 is phosphorylated on Ser910 and Ser935 following activation of the inflammatory toll-like receptor pathway and inflammatory cytokines are often increased in PD patients. Whether LRRK2 protein or phosphorylation is altered in idiopathic PD is unknown. We therefore measured LRRK2 protein and its phosphorylation in peripheral blood mononuclear cells (PBMCs) from 33 idiopathic Parkinson's disease patients and 27 age-matched controls. We found no significant difference in total LRRK2 protein levels in PBMCs from PD patients compared to controls. Furthermore, total LRRK2 protein expression was not effected by age, disease duration, disease severity or levodopa medication. The amount of phosphorylation on LRRK2 at both Ser910 and Ser935 correlated highly with total LRRK2 levels and was also unchanged in PD patients. Therefore, changes in LRRK2 Ser910/Ser935 phosphorylation in PBMCs are unlikely to contribute to idiopathic Parkinson's disease or be of utility as a disease biomarker. However, the invariance of Ser910 and Ser935 phosphorylation in PD PBMC's suggests that these residues could be used as pharmacodynamic biomarkers for the effectiveness of LRRK2 kinase inhibitors in patients.

Key words: Kinase, phosphorylation, monocyte, lymphocyte, inflammation, antibody.

LRRK2, Parkinson's disease, neurodegeneration, immunity

Introduction

Genetic mutations in the Leucine-Rich Repeat Kinase 2 (LRRK2) enzyme are considered a leading cause of autosomal dominant familial Parkinson's disease (PD) [1,2]. LRRK2 is a large (280kDa) protein unusual in that it contains both active GTPase and kinase catalytic domains. Six validated pathogenic mutations have been reported for LRRK2 with three mutations occurring in the GTPase domain (R1441C/G/H), one mutation occurring in the C-terminal of RAS (COR) domain (Y1699C) that adjoins the GTPase domain, and two mutations in the kinase domain (G2019S, I2020T) [3,4]. The most common pathogenic mutation (G2019S) resides in the magnesium binding motif of the protein kinase domain and results in an increase in LRRK2 kinase activity of 2-3 fold [5,6]. This has led to much interest in the generation of LRRK2 kinase inhibitors as potential PD therapeutics. First generation selective LRRK2 kinase inhibitors such as LRRK2-IN1 [7] and CZC25146 [8] have been employed to study cellular and pre-clinical models of PD.

Unlike G2019S, pathogenic mutations that reside in the GTPase/COR domains of LRRK2 (R1441C, R1441G, R1441H, Y1699C) reduce LRRK2 GTPase activity [9-11]. Intriguingly, these mutations also markedly reduce the phosphorylation of LRRK2 on two serine residues, Ser910 and Ser935. These sites, located just prior to the leucine-rich repeat domain, are constitutively phosphorylated and required for the binding of LRRK2 to 14-3-3 family proteins [12,13]. In addition to mutations, treatment of cells with LRRK2 kinase inhibitors also results in dephosphorylation of LRRK2 and concomitant disassociation of LRRK2 and 14-3-3 [7,14]. Despite being regulated by LRRK2 kinase activity, neither Ser910 nor Ser935

are direct LRRK2 autophosphorylation sites [14,15]. Indeed the kinase(s) and/or phosphatases that regulate LRRK2 Ser910/Ser935 phosphorylation are currently unknown. Nonetheless, this phenomenon has found widespread utility as an *in vivo* biomarker for LRRK2 kinase inhibitor function.

A number of recent reports have demonstrated a robust expression of LRRK2 in circulating immune cells. In particular, B-lymphocytes and monocytes have a high expression of LRRK2 with T-lymphocytes a lower or negligible expression of LRRK2 [16-18]. The expression of LRRK2 in immune cells can be further increased by interferon gamma [16] a cytokine previously implicated in neuroinflammation and PD [19,20]. Bacterial lipopolysaccharide (LPS), an agonist of inflammatory signaling mediated via toll-like receptor (TLR) 4, has been reported to both increase [21] and decrease [22] LRRK2 protein expression. Furthermore activation of TLR pathways can result in increased phosphorylation of LRRK2 on Ser910 and Ser935 [23]. However, whether endogenous LRRK2 or its phosphorylation is altered in idiopathic PD has not been explored. Such alterations could comprise disease biomarkers and/or provide further insight into the role of LRRK2 in idiopathic PD.

Methods

Subjects

Sixty subjects were recruited with informed consent and the study was approved by the Macquarie University Human Research Ethics Committee (reference 5201100874).

Demographic data are presented in Table 1. To focus on changes early in PD progression, subjects with less than 5 years disease onset were chosen. Patients with a strong family history or early onset PD were excluded and no patient or control subject had an immune, inflammatory or neurological disorder other than PD. Clinical severity of PD was assessed by

the Hoehn and Yahr scale [24] and the Movements Disorders Society Unified Parkinson's Disease Rating Scale (MDS-UDPRS) [25]. Use of dopamine replacement medication was recorded and levodopa equivalent dose calculated [26].

Peripheral blood mononuclear cell (PBMC) isolation

Blood was collected into sodium heparin cell preparation tube vacutainers (BD Biosciences, San Jose, CA) between the hours of 9 am and 11 am and then centrifuged at 1600 x g for 15 min in a swing bucket rotor (Multifuge 3SR, Thermofisher). The layer of PBMCs was removed and diluted with 2 volumes of Dulbecco's Modified Eagle Medium (DMEM) before being pelleted by centrifugation at 200 x g for 15 min to reduce platelet contamination. The supernatant was discarded and PBMCs resuspended in 5 ml DMEM for cell counting and viability using trypan blue exclusion and an automated cell counter (Countess, Life Technology).

Genotyping

Genomic DNA was extracted from 2×10^5 PBMCs using a spin column miniprep kit (Thermo Fisher Scientific). Restriction fragment length polymorphism analysis was used to genotype PD patients and controls for LRRK2 G2019S and R1441G mutations using previously published primers, PCR conditions and restriction enzymes [1,27,28].

Cell lysis and immunoblot

2×10^6 cells were removed and pelleted by centrifugation at 300 x g for 5 min. Cell pellets were lysed in buffer containing 50 mM Tris HCL pH 7.5, 1 mM EGTA, 1 mM EDTA, 1 mM sodium orthovanadate, 50 mM sodium fluoride, 5 mM sodium pyrophosphate, 0.27 M sucrose, 1 mM benzamidine, 1 mM PMSF and 1% (v/v) Triton X-100. Lysates were clarified

by centrifugation at 13,000 x g for 20 min and snap frozen in liquid nitrogen. Samples were stored at -80°C until patient sample collection was completed. Lysates were then thawed on ice and protein concentration determined by BCA assay (Pierce). 15 µg lysate was resolved on 4-12% Novex Tris-glycine gels (Life Technology) and transferred to nitrocellulose membrane (Biorad). Membranes were blocked with 5% skimmed milk powder in Tris buffered saline with 0.1% (v/v) Tween 20 (TBST). Membranes were probed overnight for LRRK2 and phosphorylated LRRK2 using rabbit monoclonal antibodies described and validated previously [23]. Membranes were also blotted for beta-actin to control for any differences in loading. Anti-rabbit and anti-mouse horseradish peroxidase (HRP) secondary antibodies (Biorad) were used to detect immune complexes using enhanced chemiluminescence reagent (GE Healthcare). Images were scanned and then quantitated using Image J [29].

Statistics

Statistical analyses were performed using SPSS (IBM SPSS Statistics 18, Somers, New York, USA) and a *p* value less than 0.05 accepted as significant. Multivariate analysis was used to identify any differences in protein levels between groups co-varying for age. The mean difference and standard error are given for all values. Spearman correlations were used to determine any relationship between LRRK2 levels as well as between LRRK2 levels and demographic variables (see Table 1).

Results

LRRK2 protein expression and phosphorylation in idiopathic PD

To determine whether peripheral LRRK2 expression or phosphorylation is altered in patients with idiopathic PD, immunoblot analysis was performed using peripheral blood mononuclear

cells freshly obtained from 33 PD patients and 27 age-matched controls (Table 1). Similar numbers of PBMC's were obtained from blood samples for both groups and cell viability was equally high (Table 1). All subjects were negative for LRRK2 G2019S and R1441G mutations (data not shown). Protein levels of LRRK2 and phosphorylated LRRK2 were normalized to the expression of beta-actin, which was not changed between control and PD groups (Fig 1). The complete set of immunoblots used for quantitation are shown in supplementary figure S1. Multivariate analysis of the data showed no changes in either total LRRK2 (Fig 2A), or LRRK2 phosphorylated at Ser910 (Fig 2B), or LRRK2 phosphorylated at Ser935 (Fig 2C). The immunoblots used for quantitation are shown in supplementary figures S1. The ratio of Ser935 phosphorylated to total LRRK2 (Fig 2D) was also the same with PD compared to controls. Both LRRK2 Ser910 and LRRK2 Ser935 phosphorylation correlated with total LRRK2 expression (Fig 2E and Fig 2F) however, in patients with PD there was no correlation between either phosphorylated or total LRRK2 and disease duration, disease severity (as assessed by either Hoehn and Yahr or UDPRS) or with daily levodopa (or equivalent) dose.

Discussion

Discovered in 2004 as the PARK8 locus responsible for autosomal dominant PD [1,2], there has been much interest in the potential of LRRK2 as a therapeutic target for the disease. This interest has largely centered around the kinase activity of LRRK2 as the most common pathogenic mutation, G2019S, increases the kinase activity of LRRK2 [5,6]. Expression of G2019S LRRK2 has toxic effects on patient derived cellular models [30-33] and overexpression induces behavioral deficits and neurodegeneration in *C.elegans* and *Drosophila* models [34,35]. Importantly, these effects can be largely overturned with inhibitors of LRRK2 kinase activity [31-35]. Whilst promising for PD patients harboring

LRRK2 G2019S mutations, little is known about the contribution of wild type LRRK2 to the more common idiopathic form of PD. In this study we show that there are no global changes in patients with idiopathic PD and less than 5 years disease onset, in either the level of LRRK2 protein, or in its phosphorylation at Ser910 and Ser935 in PBMCs, cells that highly express the protein.

There has been much interest surrounding the LRRK2 Ser910 and Ser935 phosphorylation sites. In cellular overexpression systems the pathogenic R1441C, R1441H, R1441G, Y1699C and I2020T mutations result in a loss of phosphorylation of LRRK2 at Ser910 and Ser935, suggesting a potential role in PD pathogenesis for these residues [12]. The phosphorylation of these residues is required for binding of LRRK2 to members of the 14-3-3 family of adaptor proteins [12,13]. In cell culture models, mutations ablating LRRK2 phosphorylation and 14-3-3 binding strikingly alter the localization of LRRK2 from predominantly cytoplasmic, to inclusions resembling cytoskeletal structures [12,36]. However, the extent to which this occurs with endogenous LRRK2 remains to be validated. In contrast, to other pathogenic mutations, G2019S does not alter the phosphorylation of LRRK2 at Ser910/Ser935 in overexpression models or immortalized lymphoblasts from G2019S mutation carriers [14]. Moreover, treatment of cells with inhibitors of LRRK2 kinase activity also results in a loss of endogenous Ser910/Ser935 phosphorylation and subsequent 14-3-3 binding [7,14]. Dephosphorylation of these LRRK2 sites with kinase inhibitors occurs in a dose-dependent manner and observation serves as a control for LRRK2 kinase inhibitors employed for *in vivo* and *in vitro* modeling. Indeed such studies have suggested novel neuroprotective strategies for the treatment of PD [32,34]. Our results show that the phosphorylation of LRRK2 at Ser910 and Ser935 correlates with the expression of total LRRK2 protein in PBMCs from idiopathic PD patients, with all remaining unchanged by disease indices or medication. This suggests

that loss of phosphorylation of LRRK2, at least in PBMCs, is unlikely to contribute to idiopathic PD pathogenesis and will not constitute a readily available disease biomarker for idiopathic PD. When assays become available, further studies will be required to directly assess LRRK2 kinase activity in PD patients. Intriguingly, that the phosphorylation of LRRK2 at Ser910/Ser935 was unchanged with any of the relevant PD factors measured, suggests that measuring these residues in PBMCs could be appropriate to monitor LRRK2 inhibitor effectiveness in humans.

As well as PD, mutations in LRRK2 have also been associated with an increased risk of Crohn's disease [37,38], an inflammatory disorder of the bowel. One such mutation, M2397T, reportedly results in a lower expression of LRRK2 protein in patient B-lymphocytes [22]. Furthermore, macrophages from LRRK2 deficient mice secrete higher levels of inflammatory cytokines following specific stimuli, rendering LRRK2 null mice more susceptible to inflammatory bowel disease [22]. A number of studies have also uncovered higher peripheral inflammatory cytokines in PD patients (for review see [39,40]) however, our results of no difference in LRRK2 protein between idiopathic PD and controls suggest that any increased peripheral inflammatory response in PD is not mediated via reduced levels of LRRK2 in PBMCs. As PBMCs comprise a number of different immune cells however, our results cannot rule out specific changes in LRRK2 expression or phosphorylation in distinct immune cell subsets. For example LRRK2 is expressed highest in CD14⁺CD16⁺ monocytes [17]. This monocyte subpopulation, which constitutes less than 10% of blood monocytes and therefore around 1% of PBMCs, is considered the most important for secretion of proinflammatory cytokines [41]. The study of LRRK2 phosphorylation in distinct immune cell subsets requires advances in flow cytometry compatible antibodies.

Overall our study has shown that the levels of LRRK2 and its phosphorylation at Ser910 and Ser935 remain unchanged in patients with idiopathic PD. The measurement of phosphorylation of LRRK2 at these sites in PBMCs can therefore be used as a pharmacodynamic tool for the efficacy of LRRK2 inhibitors currently under development for the treatment of PD. However, further work is required to directly assess LRRK2 kinase activity and ascertain any consequence of long-term dephosphorylation of LRRK2 in peripheral immune cells induced by LRRK2 kinase inhibitors.

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Conflicts of interest

The authors have no conflict of interest to report.

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Table 1 Demographic data

	Control	Parkinson's Disease
Number	27	33
Age (years)	68 ± 1.2	69 ± 1.3
Sex	8M / 19F	22M / 11F
Disease Duration (months)	-	45 ± 4.9
H&Y	-	1.8 ± 0.1
UPDRS	-	15.4 ± 1.5
PBMC yield	$1.74 \times 10^7 \pm 1.54 \times 10^6$	$1.79 \times 10^7 \pm 1.48 \times 10^6$
PBMC viability	$92\% \pm 1.0$	$91\% \pm 1.1$

PBMC=peripheral blood mononuclear cells, H&Y=Hoehn and Yahr score [24].

UPDRS=Unified Parkinson's Disease Rating Scale [25].

FIGURE LEGENDS

Figure 1. Immunoblot of LRRK2 expression and phosphorylation in PBMC's

Representative immunoblots for beta-actin, LRRK2 and LRRK2 phosphorylated at serines 910 and 935. Parkinson's disease patients are represented with an asterisk. The complete set of immunoblots is presented in supplementary figure 1.

Figure 1

Figure 2. Quantitation of LRRK2 expression and phosphorylation in PBMC's

Peripheral blood mononuclear cells were isolated from 27 control and 33 idiopathic PD patients and protein lysates immunoblotted for LRRK2 protein (A), LRRK2 phosphorylated at serine 910 (B) or LRRK2 phosphorylated at serine 935 (C). The ratio of LRRK2 phosphorylated at serine 935 to total LRRK2 was also determined (D). All immunoblots were normalized to beta-actin. Results are presented as individual data points and also mean $\pm$ SEM. The correlation between LRRK2 protein and its phosphorylation at serine 910 (E) and 935 (F) was determined for all subjects.

Figure 2

SUPPLEMENTAL FIGURE LEGEND

Figure S1. LRRK2 expression and phosphorylation in PD patients and controls

Immunoblots for beta-actin, LRRK2 and LRRK2 phosphorylated at serines 910 and 935.

Parkinson's disease patients are represented with an asterisk.

Supplementary Figure 1