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Leucine-rich repeat kinase 2 modulates cyclooxygenase 2 and the inflammatory response in idiopathic and genetic Parkinson's disease

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ABSTRACT

Inflammatory mechanisms are activated in aging and late-onset neurodegenerative diseases, such as Parkinson's disease (PD). Mutations in leucine-rich repeat kinase 2 (LRRK2) contribute to both idiopathic and familial forms of PD. Here, we investigated the involvement of LRRK2 in inflammatory pathways using primary dermal fibroblasts from patients with 2 common mutations in LRRK2 (G2019S and R1441G), idiopathic PD and age-matched healthy individuals. Basal cyclooxygenase (COX)-2 RNA levels were very high in the fibroblasts of all patients. Remarkably, LRRK2 silencing experiments significantly reduced basal COX-2 levels and COX-2 induction after a pro-inflammatory stimulus. Additionally, in samples from patients with the R1441G mutation and with idiopathic PD, we found a prominent cytoplasmic re-distribution of human antigen R, a protein that, among others, stabilizes COX-2 RNA. Furthermore, the response to lipopolysaccharide was defective in these 2 groups, which showed weak induction of pro-inflammatory cytokines and reduced NFκB transcriptional activation. In summary, we describe multiple defects in inflammatory pathways in which LRRK2 appears to be critically involved. Further studies are required to establish the therapeutic implications of inflammatory dysregulation in the pathophysiology of Parkinson's disease.

1. Introduction

Parkinson's disease (PD) is the second most common neurodegenerative disease, with age as the most influential factor determining the onset and progression of both idiopathic and familial forms of the disease. Recent evidence has demonstrated that inflammation is a fundamental process contributing to the death of dopamine neurons characteristic of PD (Collins et al., 2012; Hirsch et al., 2012; Phani et al., 2012). Mutations in leucine-rich repeat kinase 2 (*LRRK2, PARK8*) are found in 6%–40% of familial PD cases and are the genetic factor with the highest prevalence in late-onset sporadic PD (~3%) (Paisan-Ruiz et al., 2004; Zimprich et al., 2004). Although brain pathology has shown variability (Zimprich et al., 2004), including cases with no Lewy bodies and prominent tau pathology (Hasegawa et al., 2009; Marti-Masso et al., 2009), clinical features are indistinguishable from late-onset sporadic PD. Therefore, there is hope that understanding LRRK2-associated pathogenesis will provide valuable insight into the biological mechanisms operating also in the sporadic forms of the disease.

The *LRRK2* gene encodes a large protein that has multiple domains, including a Roc GTPase and a kinase catalytic domain (Paisan-Ruiz et al., 2004). Initially, it was hypothesized that mutations in LRRK2 cause disease through a toxic gain-of-function mechanism (Greggio et al., 2009; Li and Beal, 2005). However, only the G2019S mutation robustly increases kinase activity in vitro and the contribution of catalytic activities to LRRK2 pathobiology is poorly defined.

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In the brain, LRRK2 is expressed at low levels in neurons, astrocytes, and microglia, and there is no apparent correlation between cellular or regional expression and susceptibility to disease process (Sharma et al., 2011). LRRK2 is also expressed in peripheral tissues, in particular in kidney and lung, and in cells of the immune system (Santpere and Ferrer, 2009). Although it is increasingly clear that inflammatory mechanisms contribute to the neuronal degeneration occurring in PD, these are poorly understood (Collins et al., 2012; Hirsch et al., 2012; Phani et al., 2012). Intriguingly, polymorphisms in LRRK2 are associated with inflammatory bowel disease (Barrett et al., 2008; Franke et al., 2010; Umeno et al., 2011), and growing evidence suggests that LRRK2 plays a role in innate immunity and modulates NFκB pathway (Dzamko et al., 2012; Gardet et al., 2010; Gillardon et al., 2012; Kim et al., 2012; Liu et al., 2011). Cyclooxygenase (COX)-2, the rate-limiting enzyme in the synthesis of prostaglandins, plays a central role in these pathways, yet a link between LRRK2 and COX-2 has not been investigated.

Fibroblasts are not affected in PD but provide an excellent cellular model for examining the involvement of wildtype and mutated endogenous proteins in major signaling pathways (Hoepken et al., 2008; Mortiboys et al., 2010). Moreover, in fibroblasts from aged patients, the accumulating effects of polygenic factors and environmental insults over the patient's lifespan are preserved. The applicability of this model is further underscored by the recent validation of therapeutic targets identified in PD fibroblasts with parkin and LRRK2 mutations (Mortiboys et al., 2013). We previously identified an extracellular signal-regulated kinases (ERK)-mediated increase in autophagy in LRRK2^{G2019S} fibroblasts (Bravo-San Pedro et al., 2013). Here, we report a remarkable increase in COX-2 RNA levels in PD, and a blunted response to a pro-inflammatory stimulus. Our results show that LRRK2 is indeed involved in the inflammatory response and that this pathway is dysregulated in PD.

2. Methods

2.1. Human samples

Skin samples were obtained from patients diagnosed at the Movement Disorders Unit of Hospital Donostia and from healthy individuals (Table 1). All subjects gave informed consent using forms approved by the Ethical Committee on the Use of Human Subjects in Research at Hospital Donostia. A genetic analysis performed at the Biodonostia Institute confirmed the presence of the mutations. Some patients have been included in previous publications (Bravo-San Pedro et al., 2013; Ruiz-Martinez et al., 2010). Skin fibroblasts were isolated at the Cell Culture Unit of the Biodonostia Institute as described (Bravo-San Pedro et al., 2013). Fibroblasts were cultivated in Dulbecco's Modified Eagle Medium (high glucose modification, Sigma-Aldrich San Louis, MO) with 10% fetal bovine serum, 2 mM L-glutamine, and antibiotics. Primary cells under 20 passages were used for all experiments. Morphologic features and growth rate were normal and comparable between samples during the time of the study. Representative images of cells used in the experiments are provided in Supplementary Fig. 1.

Table 1
Human dermal samples

Group	n	Male:female ratio	Age at the time of the biopsy (y)
Control	6	4:2	52–63
LRRK2^{G2019S}	4	3:1	60–67
LRRK2^{R1441G}	5	2:3	62–82
iPD	3	1:2	64–82

Subject information for control, mutant LRRK2 and iPD cases used for analytical determinations in fibroblast samples.
Key: iPD, idiopathic Parkinson's disease.

2.2. LRRK2 gene knockdown

LRRK2 expression was stably silenced in fibroblasts using commercially available small hairpin RNA sequences cloned into the pLKO.1 vector (TRCN0000368513, TRCN0000358256, and TRCN0000358257 MISSION small hairpin RNAs, Sigma-Aldrich). Lentiviral particles were produced and titrated at the viral vector platform in Inbiomed according to standard protocols. Transduction was performed in suspension with a multiplicity of infection of 10. All experiments were performed 5 days after transduction.

2.3. Western blotting

Whole-cell lysates were prepared in radioimmunoprecipitation (RIPA) assay buffer (50 mM Tris-HCl, 150 mM NaCl, 1% NP-40, 0.25% sodium deoxycholate, 0.1% SDS, 1 mM EDTA, 1 mM sodium orthovanadate, 1 mM NaF) with a protease inhibitor cocktail (Roche, Basel, Switzerland). Sodium dodecyl sulfate polyacrylamide gel electrophoresis and protein transfer and blotting were carried out according to standard procedures. Primary antibodies are listed in Supplementary Table 1. Visualization of horseradish peroxidase-labeled proteins was performed using enzyme-linked chemifluorescence (ThermoFisher Scientific, Waltham, MA) and quantified using ImageJ software 1.42q (NIH, Bethesda, MD). Levels of phosphorylated proteins were normalized to total levels of the respective kinase.

2.4. Immunofluorescence

Cells cultured on glass coverslips were fixed with 4% paraformaldehyde and incubated overnight at 4 °C with primary antibodies (Supplementary Table 1) followed by appropriate Alexa-fluorescent secondary antibodies. Images were acquired in a Zeiss LSM510 confocal microscope and analyzed using ImageJ (NIH). Cells from 2 different individuals per group were used in each experiment and at least 2 experiments were averaged for analyses. All counts were performed blind to the identity of the sample. Human antigen R protein (HuR) subcellular localization was scored on images acquired at a 40× magnification in randomly selected fields; on average 150 cells per sample were analyzed in a total number of 10 fields. Cells were classified into 3 categories according to the predominant localization of HuR signal: (1) "nuclear", fully delineating the nuclear profile; (2) "cytoplasmic", completely excluded from the nucleus; and (3) "both" (for representative examples see figures). For p65 analysis, images were acquired with a 20× objective in randomly selected fields and on average 200 cells per sample were analyzed in a total number of 5 fields; the presence or absence of nuclear signal was scored.

2.5. Real-time reverse transcription polymerase chain reaction

Total RNA was extracted using Trizol total RNA isolation reagent (Gibco, Life Technologies, Carlsbad, CA) and the RNeasy Qiaprep (Qiagen, Hilden, Germany). Complementary DNA was synthesized from total RNA using random hexamers according to the *GeneAmp* RNA PCR *Core* Kit (Life Technologies) and the high-capacity complementary DNA RT kit (Applied Biosystems Life Technologies). Real-time PCR was performed using the StepOne Detection System (Applied Biosystems). Comparative analysis of gene expression levels (ΔΔCt) was carried out using GAPDH as the reference. Primer sequences are listed in Supplementary Table 2.