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Oxidative stress alters the regulatory control of p66^{Shc} and Akt in PINK1 deficient cells

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ABSTRACT

Mitochondrial dysfunction is involved in the underlying pathology of Parkinson's Disease (PD). PINK1 deficiency, which gives rise to familial early-onset PD, is associated with this dysfunction as well as increased oxidative stress. We have established primary fibroblast cell lines from two patients with PD who carry mutations in the PINK1 gene. The phosphorylation of Akt is abrogated in the presence of oxidative stressors in the complete absence of PINK1 suggesting enhanced apoptotic signalling. We have found an imbalance between the production of reactive oxygen species where the capacity of the cell to remove these toxins by anti-oxidative enzymes is greatly reduced. The expression levels of the anti-oxidant enzymes glutathione peroxidase-1, MnSOD, peroxiredoxin-3 and thioredoxin-2 were diminished. The p66^{Shc} adaptor protein has recently been identified to become activated by oxidative stress by phosphorylation at residue Ser36 which then translocates to the mitochondrial inner membrane space. The phosphorylation of p66^{Shc} at Ser36 is significantly increased in PINK1 deficient cell lines under normal tissue culture conditions, further still in the presence of compounds which elicit oxidative stress. The stable transfection of PINK1 in the fibroblasts which display the null phenotype ameliorates the hyper-phosphorylation of p66^{Shc}.

1. Introduction

Biochemical abnormalities such as oxidative stress and cell death via the impairment of energy metabolism have long been linked to Parkinson's Disease (PD). The implication of mitochondrial dysfunction in the pathogenesis of PD was first alluded to upon the realization that a number of patients with Parkinsonian syndrome had been accidentally exposed to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). MPTP was found to be toxic to dopaminergic neurons and its conversion to MPP+ is an inhibitor of complex I of the respiratory chain [1]. It was later found that complex I activity is reduced in post-mortem substantia nigra of PD patients [2].

Recently, PD-associated genes which interact with the mitochondrial have been identified, Parkin, DJ1 and PTEN induced kinase (PINK1). Both DJ1 and Parkin can rescue mitochondrial abnormalities caused by the loss of PINK1 [3,4]. The PINK1 gene product is the only protein with a clear mitochondrial targeting sequence and its mutation has been shown to cause early-onset PD with autosomal recessive inheritance [5]. The cellular location and function of the PINK1 protein remains controversial. Compelling evidence suggests that it is located within the outer mitochondrial membrane with the C-terminal serine/threonine kinase domain facing the cytoplasm and N-terminal tail inside the mitochondria [6]. The exact function of PINK1 remains an enigma, but it is clear that the protein is involved in the regulation of mitochondrial morphology [7,8] and provides protection against oxidative stress [9–12].

Shc growth factor adaptor proteins act as a conduit between cell receptors and down stream cellular signalling. There are three isoforms, p46, p52 and p66. Phosphorylated p46/p52 proteins associate with Grb2 and SOS to activate the Ras pathway leading to cell proliferation. The p66^{Shc} isoform has been shown to interfere with this pathway cell survival pathway [13] and has been recently been recognized as a mediator of oxidative stress and mitochondrial dysfunction. Oxidative stress has been shown to induce PKCβ phosphorylation of p66^{Shc} at serine-36 upon which the protein complexes with the protein Pin1 and translocates to the inner mitochondrial membrane space leading to apoptosis [14–17].

The objective of this study was to investigate the possibility that the protection invoked by PINK1 operates through the p66^{Shc} signalling pathway. We have collected fibroblasts from two patients with early-onset PD and molecular defects in the PINK1 gene. Included in these investigation are two patient cell lines with mutation in PTEN which induces the transcription of PINK1. These cells lines were exposed to various oxidative stressors. We have found a clear correlation to the absence or mutation of both PINK1

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and PTEN and the phosphorylation state of p66. The changes to the phosphorylation state of p66 is ameliorated upon transfection of the mutant PINK1 cell lines with the wild-type protein.

2. Methods and materials

2.1. Reagents

All reagents were purchased from Sigma–Aldrich unless otherwise stated. Cultured skin fibroblasts were grown from forearm skin biopsies (taken with informed consent) in α-MEM culture medium (11 mm glucose) and 10% fetal calf serum (Wisent, Inc., Saint-Jean-Baptiste deRouville, Quebec). Patient PINK1 A is male and has pathogenic compound heterozygous mutations p.E240K and p.L489P. PINK1 B patient is male and has a homozygous nonsense mutation in the protein coding region c.1366C>T which causes an early stop and results in a truncated protein. The two patients with PTEN protein mutation are both heterozygous for the nonsense p.R130X truncated protein. PTEN acts as a tumour suppressor protein and its defect gives rise to autosomal dominant inheritance of PTEN Hamartoma Tumour Syndrome. Age matched control fibroblast cell lines (juvenile and mature controls for PTEN and PINK1 patients, respectively) were purchased from Coriell Cell Repository, Camden, NJ.

2.2. Transformed cell lines

Control, PINK1A and PINK1B cell lines were immortalized with a plasmid containing SV40 large T antigen [18] for the creation of stable cell lines to be transfected with a PINK1 expression vector (Superfect, Invitrogen, Carlsbad, CA). The expression vector contained the full length PINK1 gene which was ligated into pcDNA3.1(+) vector (Invitrogen). Briefly, PINK1 RNA was isolated from control skin fibroblasts, cDNA was generated from the RNA using random primers and full length coding region of PINK1 was amplified by RT-PCR. The primer pairs used: Forward-CACCGCC CCAAGTTTGTTGTGA; Reverse-AGGCCTTTTCCGGCTAACCA. The PCR product was ligated into TOPO cloning vector (Invitrogen). The full length PINK1 from the TOPO vector was directionally cloned into pcDNA3.1(+) between Not1 and XbaI restriction sites. The sequence of all constructs were confirmed by fluorescent sequencing by ACTG Corporation, Toronto. The transfected cell lines were maintained in α-MEM culture medium (11 mm glucose) and 20% fetal calf serum (Wisent, Inc., Quebec) in the presence of 100 μg/ml of the antibiotic G-418 (Invitrogen).

2.3. Metabolic work-up

PINK1 A and PINK1 B cell lines were assessed for lactate/pyruvate ratio, all respiratory chain complex activities, [2-^{14}C]pyruvate whole cell oxidation, pyruvate dehydrogenase complex, alpha-ketoglutarate complex, pyruvate carboxylase as well as ATP, ADP and AMP levels were determined by standard methods [19–22].

2.4. Cell treatment

All cell lines were grown to confluency on a 10 cm tissue-culture dish. The cells were then treated by a variety of oxidative stressors: 0.5 mM H_2O_2 for 20 min; 10 μM bradykinin or 10 μM paraquat (methyl viologen) for 24 h. The cells were quickly washed with phosphate buffered saline and harvested with 300 μl of TNEN buffer containing 50 mM Tris–HCl pH 7.5, 100 mM KCl, 1 mM EDTA, 0.5% igepal, 1 mM PMSF, 1 mM sodium orthovanadate, 10 mM NaF, 1 mM β-glycerol phosphate, 10 μg/ml leupeptin and 10 μg aprotonin. The cell lysates were then snap frozen in liquid nitrogen. Mitochondria were isolated from 20 plates of treated cells [23].

2.5. Western blotting

Antibodies used were polyclonal anti-human Shc from Upstate Biotechnologies (Lake Placid, NY), monoclonal anti-human Shc/p66 (pSer^{36}) from Calbiochem (Gibbstown, NJ), polyclonal anti-mouse AKT Cell Signalling (Danvers, MA), polyclonal anti-mouse AKT (pSer^{473}) Cell Signalling, polyclonal anti-mouse p53 from Santa Cruz Biotechnology (Santa Cruz, CA), polyclonal anti-human BAX from Cell Signalling, polyclonal anti-human PKA from Santa Cruz, polyclonal anti-human PTEN from Cell Signalling, polyclonal anti-human PTEN (pSer^{380}/Thr$^{382/383}$) from Cell Signalling, polyclonal anti-human MnSOD antibody raised against peptide NWENVTERYMACKK, polyclonal anti-DJ1 was a gift from Dr. Tak Mak of the Division of Stem Cell/Developmental Biology, Ontario Cancer Institute, Toronto, polyclonal anti-human BCL2 from Zymed Laboratories (San Francisco, CA), polyclonal peroxiredoxin-3 raised against peptide SPAASKEYFQKVNQ, polyclonal thioredoxin-2 raised against peptide DEDQLEAFLKKLIG, polyclonal anti-human PINK1 raised against peptide QKSKPGPDPLDTRRIQ, polyclonal anti-human GAPDH from abcam (Cambridge, MA), polyclonal anti-bovine glutathione peroxidase-1 was raised against the purified bovine protein purchased from Sigma (Oakville, ON), monoclonal anti-B-actin antibody was purchased from Sigma (Baie d'Urfe QU).

Hundred micrograms of cell lysate or 50 μg purified mitochondria was loaded onto NuPAGE 4–12% Bis–Tris gel (Invitrogen) and electrophoresed to separate the protein bands by MW. The protein was transferred onto PVDF membrane and blocked with 5% skim milk/TBST. The membrane was probed with antibodies listed above according to manufacturers instructions. In-house antibodies were used at 1:1000 dilution and incubated with the membrane overnight in 3% skim milk/TBST at 4 °C. Immunoreactive proteins were visualized using Western Lightning Chemiluminescence Reagent Plus (PerkinElmer Life and Analytical Sciences).

3. Results

3.1. Mitochondrial enzyme activity screen of PINK1 deficient fibroblasts

The respiratory chain complexes I–IV were assayed for activity, the levels of cellular ATP, ADP and AMP calculated, [2-^{14}C]pyruvate whole cell oxidation, pyruvate dehydrogenase complex, alpha-ketoglutarate complex and the lactate/pyruvate ratio was determined for PINK1 A and B fibroblasts. With the exception of elevated total lactate and pyruvate produced from glycolysis, all metabolites and activities tested including lactate/pyruvate ratio were within the normal range (data not shown). Total lactate and pyruvate for PINK1 A are 360.0 ± SE 23.0 and 18.0 ± SE 1.5, respectively ($N = 4$), for PINK1 B 697.7 ± SE 104.0 and 22.6 ± SE 6.6 ($N = 3$) compared to control lactate 242.8 ± SE 32.5 and control pyruvate 14.4 ± SE 1.5 ($N = 6$) all expressed as nmol/mg protein/h.

3.2. Expression Levels of p66^{Shc}, pro-apoptotic/anti-apoptotic and anti-oxidant proteins

To look for PINK1 and PTEN related changes of enzymes involved in the apoptosis/survival pathways, a variety of proteins levels which can be detected in fibroblasts were explored. We chose fibroblast cell lines from two patients with mutation in the PINK1 gene, a null-mutant (PINK1B) and a compound-heterozygote-mutant (PINK1 A) and fibroblasts isolated from two patients with identical autosomal dominant mutation in PTEN which is